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REGULATIONS

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(3) A candidate for the Degree of Doctor of Medicine, or for the Degree of Master of Surgery, shall submit for the approval of the Faculty of Medicine a thesis on any branch of knowledge comprised in or related to the curriculum for the Degrees of Bachelor of Medicine and Bachelor of Surgery, and may be required to present himself for oral or other examination in the subject-matter thereof: provided that a candidate for the Degree of Doctor of Medicine shall not submit a thesis on a subject which is exclusively surgical, and that a candidate for the Degree of Master of Surgery shall not submit a thesis on a subject which is exclusively medical.

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1. Two copies of each thesis are required. Theses must be type-written on paper approximately 10 inches by 8 inches and bound in dark cloth with stiff boards. The title of each thesis and the name of the author must be printed in block letters on the outside binding. Theses should be lodged with the Dean of the Faculty of Medicine not later than 15th September, or 15th December, or 15th March, for adjudication during the Martinmas, Candlemas, and Whitsun terms respectively.

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3. A thesis must be a dissertation written for the purpose, provided that the results of original observations already published in medical or scientific journals or in the transactions of learned societies or otherwise may be accepted in place of such a dissertation. Published papers submitted in lieu of a dissertation must be related and accompanied by a statement, preferably in the form of an introductory paper, showing the relationship between the various studies and placing the whole work critically into perspective with the general state of knowledge in the field of investigation to which the candidate's researches are related.

4. A declaration signed by the candidate that the work has been done and the thesis composed by himself must be submitted with the thesis. Where material based on work undertaken in collaboration with others is included in the thesis a separate statement clearly defining the extent of his personal contribution must also be submitted by the candidate. If the whole or any part of the subject matter of the thesis has been included in a thesis already approved for a degree in this or another University, the candidate must make a declaration to this effect and must lodge with his thesis either a copy of such previously approved thesis or a precise statement of its scope.

5. Two copies of a separate summary (500-1000 words) giving an adequate and informative abstract of the work, must be submitted with the thesis.

6. A thesis approved for the Degree of Doctor of Medicine or Master of Surgery may be deemed to be (i) Worthy of Honours or (ii) Worthy of Commendation or (iii) Sufficient.

7. If the thesis is approved, the copies submitted by the candidate shall become the property of the University."

SUMMARY

THE INFLUENCE OF LIVER FUNCTION ON THE ADRENOLYTIC AND CARCINOGENIC ACTION OF 9;10-DIMETHYL-1;2-BENZANTHRACENE.

This Thesis reports a series of investigations into the carcinogenic and adrenolytic effect of the polycyclic hydrocarbon 9;10-dimethyl-1;2-benzanthracene in the Sprague Dawley rat. The results of the investigations support the hypothesis that the carcinogenic effect is due to 9;10-dimethyl-1;2-benzanthracene itself, whereas the adrenolytic action is produced by a metabolite of the hydrocarbon. As hepatic damage was shown to protect the adrenal glands against necrosis it appears that the metabolic conversion of 9;10-dimethyl-1;2-benzanthracene to an adrenolytic derivative is a function of the liver.

In order to assess degrees of liver damage, a liver function test was developed based on the duration of Nembutal narcosis. It was found that there was correlation between the degree of liver damage produced and the ability to protect the adrenal glands by various protective measures.

THESIS SUBMITTED TO THE UNIVERSITY OF GLASGOW

FOR THE DEGREE OF Ch.M.

by

IAN RIDDELL KERNOHAN

June, 1970.

THE INFLUENCE OF LIVER FUNCTION ON THE ADRENOLYTIC AND CARCINOGENIC ACTION OF 9;10-DIMETHYL-1;2 BENZANTHRACENE.

NOMENCLATURE.

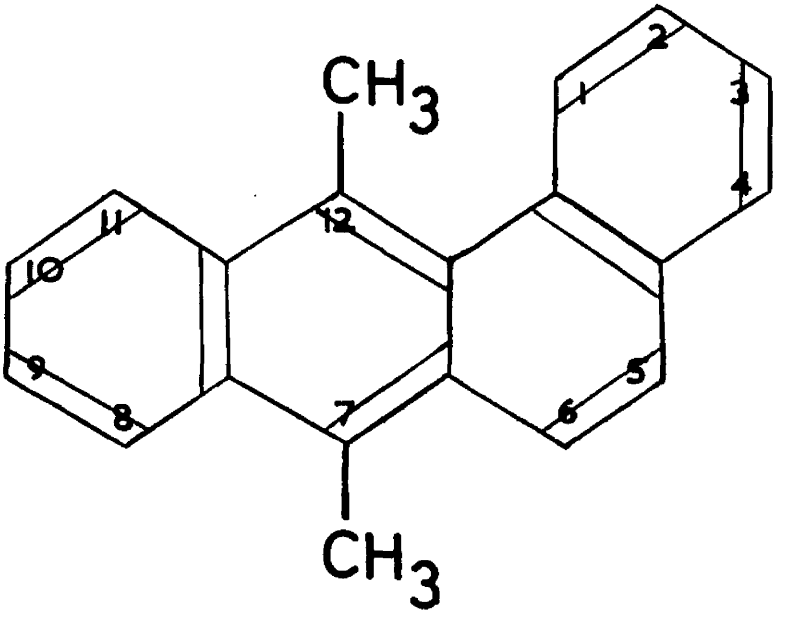
The polycyclic hydrocarbon under investigation is known both as;-

9;10 Dimethyl-1;2-Benzanthracene (British Terminology)

and

7;12-Dimethylbenz(a)anthracene (American Terminology)

For convenience the abbreviation DMBA will be used hereafter.



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INTRODUCTION.

INTRODUCTION.

Following the isolation of the polycyclic hydrocarbons these substances were found to be carcinogenic agents. In particular, DMBA proved to be extremely potent in this respect. It is now frequently used to produce mammary tumours in rats for experimental purposes. As well as its carcinogenic properties, DMBA has been found to produce massive necrosis of the adrenal glands when administered to rats. The fact that DMBA is both a carcinogenic and an adrenolytic agent has given rise to speculation concerning the relationship of these properties to each other.

Methods have already been discovered which will protect the adrenals against DMBA-induced damage. The object of this present study has been to explore the mechanism by which such protection is brought about. During the investigations, new techniques have been found which will protect the adrenal glands.

Other workers have attempted to protect animals against the carcinogenic effect of DMBA and the second part of this thesis has extended their observations by investigating the effect on tumour induction of these newly described methods for adrenal protection.

As these investigations have suggested that the liver

plays an important role in determining the biological action of DMBA, techniques to measure the metabolic activity of the liver have been developed and applied to the study of the adrenal protective mechanisms. The correlation of hepatic function with the ability to protect the adrenal glands against DMBA comprises the subject matter for the third part of this thesis.

PART I

PROTECTION AGAINST THE ADRENOLYTIC EFFECT OF DMBA.

REVIEW OF THE ADRENOLYTIC EFFECT OF DMBA.

During routine necropsies on rats which had been treated with DMBA a few weeks previously to induce mammary tumours, it was noted that the adrenal glands were calcified; the adrenal glands of animals which had received other hydrocarbons were not calcified (Huggins and Morii, 1961). Following this initial observation these workers found that DMBA would invariably produce adrenal apoplexy and necrosis soon after the administration of the hydrocarbon. Further investigations (Huggins and Morii, 1961; Morii and Huggins, 1962) into this property were undertaken and their findings were summarised as follows:-

(a) Maximal damage with haemorrhage and necrosis of zona fasciculata and zona reticularis was present on the third day following DMBA administration. The medulla and zona glomerulosa were spared.

(b) A dose of 5 mg. DMBA administered intravenously produced adrenal necrosis in all the rats treated.

(c) Oral administration required a greater dose to achieve the same incidence of adrenal necrosis.

(d) Rats aged less than 45 - 50 days were resistant to the production of adrenal necrosis.

(e) Male animals were also susceptible to the adrenolytic effect of this hydrocarbon.

(f) Different strains of rat varied in their susceptibility.

(g) Hypophysectomised rats did not develop adrenal necrosis; this resistance was abolished by the administration of adrenocorticotrophic hormone.

(h) The entire molecule of the hydrocarbon was necessary for the possession of its adrenolytic property.

Although it has been pointed out that other agents such as hexadimethrine are capable of producing adrenal necrosis, the lesion produced by DMBA does have a characteristic histological pattern (Huggins and Sugiyama, 1965).

Following the demonstration that the susceptibility of the adrenals was related to their content of corticosterone (Morii and Huggins, 1962) Metopirone was tested. This substance inhibits 11β hydroxylation and therefore interferes with the synthesis of cortisol and corticosterone. It was found to protect the adrenal glands against massive necrosis and thereby established for the first time that a mechanism existed which would prevent the adrenolytic effect of DMBA. This observation supported the hypothesis that the adrenals of immature or hypophysectomised rats were not susceptible to DMBA because of their inability to synthesize corticosterone (Currie, Helfenstein, and Young, 1962).

Another protective measure was discovered when it was

noted that treatment with 3-methylcholanthrene prior to DMBA not only lowered the corticosterone content of rat adrenal glands but prevented the development of massive adrenal necrosis (Dao and Tanaka, 1963 i). Further investigations (Huggins, Deuel and Fukunishi, 1963; Dao and Tanaka, 1963 ii), revealed that such protection could be achieved by the administration of a number of polycyclic hydrocarbons or aromatic amines prior to DMBA. Since the intravenous administration of a lipid emulsion has also been found to be effective it is evident that the protector need not be absorbed via the intestinal tract in order to exert its influence. Phenobarbital has been found to be the least complex of the aromatics to provide protection (Huggins and Fukunishi, 1964).

Study of the mechanism by which such protection is established revealed that the protectors, including DMBA itself, all induced the synthesis of the enzyme menadione reductase in the liver (Huggins and Fukunishi, 1964). Administration of dl-ethionine could eliminate protection of the adrenal glands and reduce the amount of the enzyme synthesized in response to the protectors. It was also shown that the fatal toxicity of DMBA could be prevented by the same agents that protected the adrenal glands, and that this effect too was abolished by dl-ethionine (Huggins, Ford, Fukunishi and Jensen, 1964). As the administration of

DMBA brought about a decrease in the incorporation of thymidine H^3 into deoxyribonucleic acid and the effect was prevented by the prior administration of a protective dose of 3-methylcholanthrene it was concluded that protection of the adrenals required the induction of protein synthesis and an improved synthesis of deoxyribonucleic acid (Huggins and Fukunishi, 1964).

Large numbers of compounds have now been tested for their ability to act as protectors (Huggins and Fukunishi, 1964) and it has been suggested that this property is a function of two molecular attributes:

(a) geometry

(b) ability to participate in change-transfer complexes.

The most efficient protectors were found to be planar molecules similar in geometry to nucleic acid base pairs, especially if they contained four or five benzene rings (Dao, 1964).

More recently it has been demonstrated that compounds which induce detoxifying enzymes, such as benzpyrene hydroxylase, in the liver will also protect the adrenals (Wattenberg and Leong, 1965). Such compounds include phenothiazine and some of its derivatives.

When studying factors which influence adrenal protection it is desirable to use a more critical dose of DMBA so that

the protective mechanisms are not overwhelmed. It has been found that 3 mg. DMBA administered intravenously will regularly produce 65% - 75% incidence of adrenal necrosis in female Sprague-Dawley rats aged 50 days (Wheatley, Kernohan and Currie, 1966).

Although the adrenolytic effect of DMBA is a striking property it is apparent that this hydrocarbon produces widespread changes in the metabolic activity of the subject. Such effects may have to be considered during the interpretation of any experimental results concerning adrenal or tumour protection. Disorders which have been produced include:-

1. Fatal toxicity (Huggins, Ford, Fukunishi and Jensen, 1964).
2. Loss of weight and scruffy appearance (Huggins and Morii, 1961).
3. Diarrhoea (Huggins and Morii, 1961).
4. Testicular damage (Ford and Huggins, 1963; Huggins, 1963 i).
5. Leucopenia (Huggins and Morii, 1961).
6. Fall in plasma alkaline phosphatase (Huggins and Morii, 1961).
7. Fall in plasma corticosterone (Huggins, Dovel and Fukunishi, 1963; Dale and Scutchfield, 1968).
8. Haematopoietic depression (King, 1965).
9. Impairment of the response of the accessory

reproductive organs of immature male and female rats to human chorionic gonadotropin (Hipkin, 1966).

Such a variety of effects is not surprising in view of the action of DMBA on deoxyribonucleic acid synthesis (Huggins and Fukunishi, 1964).

Although DMBA has this wide range of activities it is of interest that so far only the rat has been shown to be susceptible to its adrenolytic action (Cefis and Goodall, 1965).

The demonstration that certain methods of protection were associated with an increase in liver enzymes suggested that the liver might play an important role as regards the production of adrenal necrosis by DMBA. When it was shown that carbon tetrachloride intoxication could protect the adrenals (Wheatley, 1965 - personal communication), it was decided to investigate this effect more closely.

MATERIALS AND METHODS.

ANIMALS.

Female albino rats of the Sprague-Dawley strain were used throughout the experiments to be described. The animals were supplied by the Oxfordshire Laboratory Animal Colonies (Bicester, Oxon., England), arriving in our animal house at 40 days of age. They remained under quarantine until allocated to an experimental group at 50 days of age, at which time they weighed 140 - 180 g.

When animals of less than 40 days were required they were supplied from breeding colonies of Sprague-Dawley rats maintained in our own animal house.

The rats were fed on a standard diet of tap water and Thomson Cube (with 14% dried skimmed milk) ad libitum. This commercial feed was supplied by the North-Eastern Agricultural Co-operative Society, Ltd., Aberdeen.

The animal house was maintained at a uniform temperature of 22 ± 1°C. and the rats experienced normal daylight hours.

DMBA.

The hydrocarbon was administered in the form of a lipid emulsion. The formula of this preparation was:

DMBA	0.5% w/w
Lecithin	1.2% w/w
Poloxalkol	0.3% w/w
Cottonseed Oil	15.0% w/w
Water for injection	q.s.

Thus, 1 ml. of the emulsion contained 5 mg. DMBA.

The preparation wasmanufactured and supplied by the Upjohn Company, Kalamazoo, Michigan, U.S.A.

An emulsion identical to the above, but lacking DMBA, was used as a control material in the various experiments.

The emulsions were stored in a refrigerator at 4°C. and prior to administration thorough mixing of the contents was achieved by shaking.

Administration of the emulsion was by intravenous injection into a tail vein using a 1 ml. plastic syringe (Johnson's Ethical Plastics, Ltd., Slough, Bucks., England) and a sterile disposable No. 18 needle (Steriseal). To facilitate the venepuncture the animals were placed in a plastic box heated to 40°C. for five minutes to induce vasodilatation.

CHEMICALS.

Carbon tetrachloride supplied by:-

Analar Reagent,
British Drug Houses, Ltd.,
Poole,
England.

Thioacetamide supplied by:-

Laboratory Reagent,
British Drug Houses, Ltd.,
Poole,
England.

dl-Ethionine supplied by:-

British Drug Houses, Ltd.,
Poole,
England.

Glycine supplied by:-

British Drug Houses, Ltd.,
Poole,
England.

Nembutal (Veterinary) brand of pentobarbitone sodium supplied by:-

Abbot Laboratories, Ltd.,
Argo-vet. Division,
Queenborough,
Kent,
England.

PARTIAL HEPATECTOMY.

Partial hepatectomy was carried out under ether anaesthesia according to the technique described by Higgins and Anderson in 1931. This operation removed the large median and the left lateral lobes of the liver, and thereby reduced the liver mass by approximately 70%.

For control experiments sham hepatectomies were performed. This procedure consisted of the administration of the same type of general anaesthetic and making the same surgical incision through the abdominal wall which was then sutured. There was, however, no interference with the liver.

The muscular layer of the abdominal wall was repaired with catgut and the skin incision closed with Michel clips.

Following operation the animals were returned to their cages and allowed to recover spontaneously from the effect of the anaesthetic. They resumed their normal diet immediately.

ASSESSMENT OF ADRENAL DAMAGE.

At the completion of an experiment the rats were killed by a blow on the neck. Immediate necropsy was performed, the colour and size of the adrenal glands being noted. Tissues removed for histological examination were fixed in 4% neutral buffered formaldehyde in saline for 24 hours. Following paraffin embedding section 5 thick were cut and stained with haematoxylin and eosin. Adrenal apoplexy can be detected macroscopically owing to the red, swollen appearance of the glands, but histological examination was carried out to confirm the degree of adrenal damage.

DMBA produces selective damage in the zona fasciculata and zona reticularis. The following classification of adrenal damage has been used:-

Grade I	Normal histological appearances.
Grade II	Single cell necrosis and congestion of the blood vessels within the zona reticularis.
Grade III	Focal necrosis of the zona fasciculata and zona reticularis.
Grade IV	Severe, massive necrosis involving the zona fasciculata and zona reticularis.

Although the assessment of such damage was subjective, good correlation was obtained by independent observers; occasional differences arose concerning the allocation of a

gland to Grade I or Grade II. For the purposes of statistical analysis, the glands were considered to show either massive necrosis (Grades III and IV) or not (Grades I and II). In no case did observers differ in their assessment of the presence or absence of such massive necrosis.

The histological appearances of the various grades of adrenal damage are illustrated in Plates 1 to 4.

The relationship of adrenal weight to degree of adrenal damage is shown in Tables 1 and 2 and Figure 1. Glands which show massive necrosis are 40% heavier than undamaged glands.

When recording the results of adrenal protection animals which died before there was time for adrenal damage to manifest itself were excluded.

PLATE 1.

ADRENAL NECROSIS GRADE I.

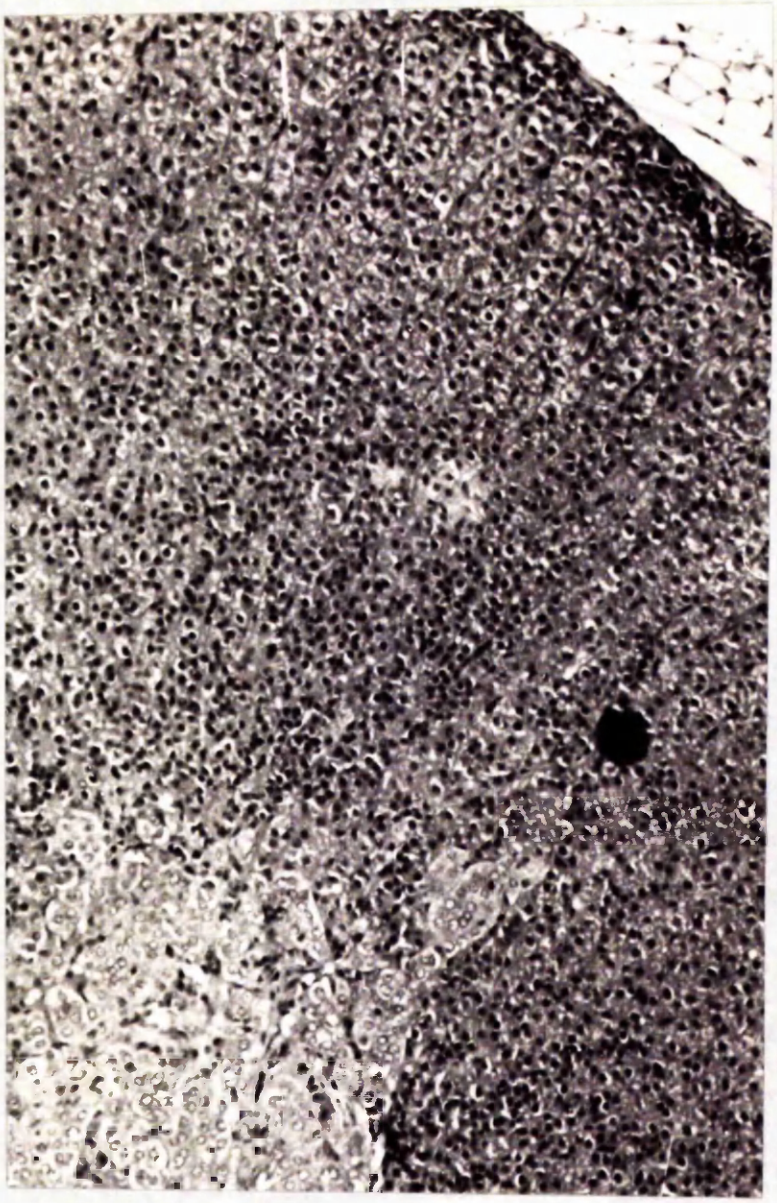


PLATE 2.

ADRENAL NECROSIS GRADE II.

PLATE 3.

ADRENAL NECROSIS GRADE III.

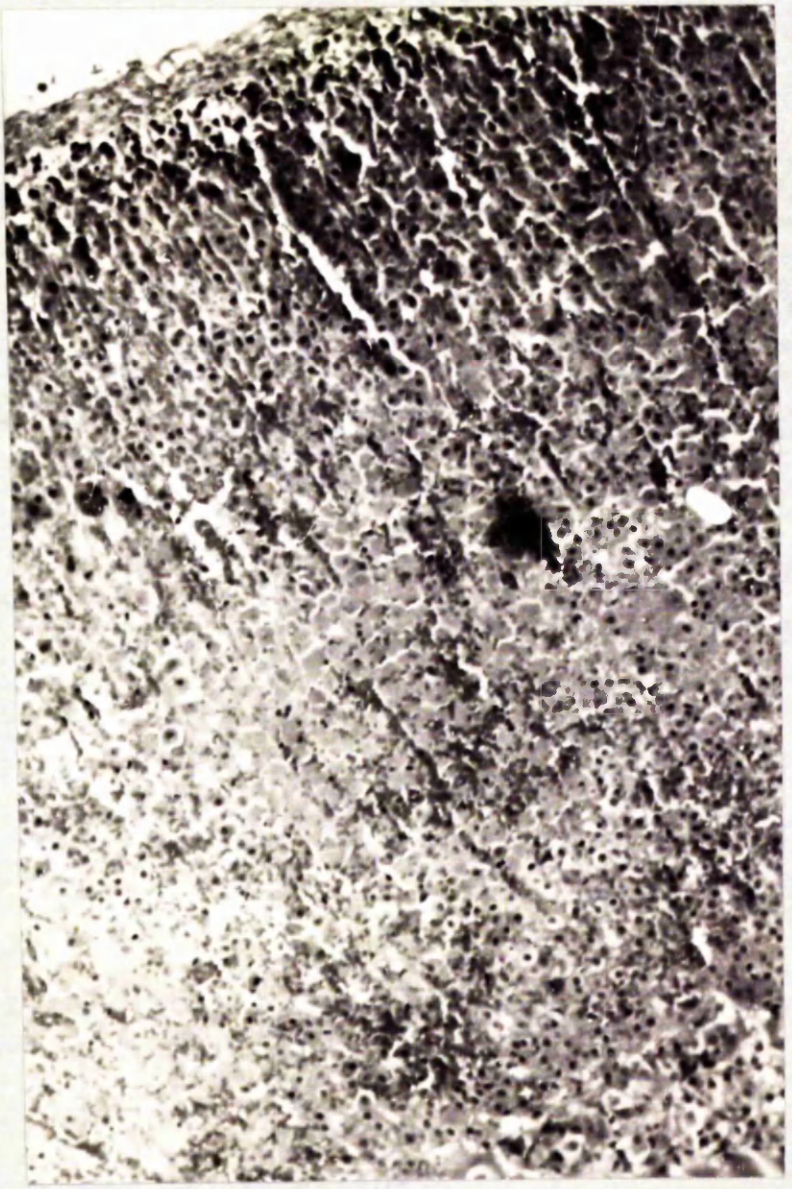


PLATE 4.

ADRENAL NECROSIS GRADE IV.

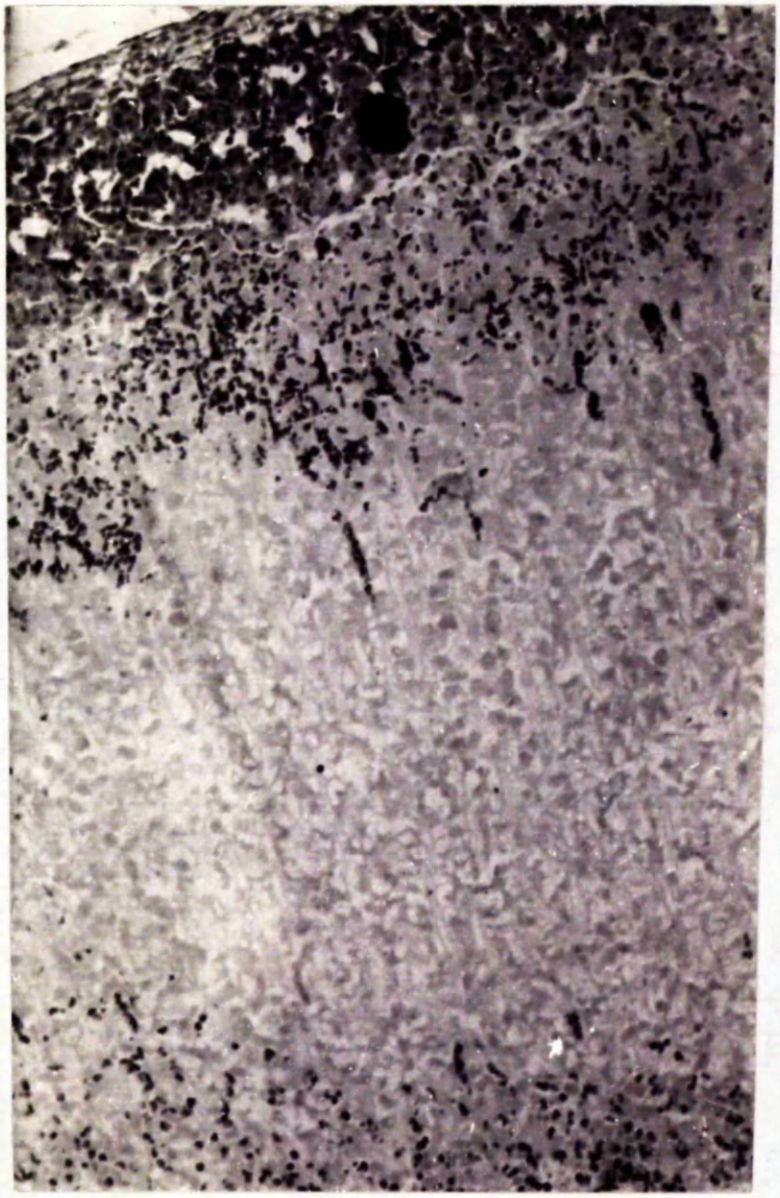


TABLE 1.

The Effect of Adrenal Necrosis on Individual Adrenal Weight.

Adrenal Weight mg.	Adrenal Necrosis Grade
25	I
28	II
25	I
26	II
33	II
21	I
24	II
51	IV
43	IV
20	I
39	IV
26	I
33	III
27	IV
37	IV
24	I
19	II
25	II
38	II
49	IV
60	IV
46	IV
40	IV
48	IV
26	IV
33	III
34	IV
31	IV
47	IV
40	IV
26	II
23	II
33	IV
39	I
27	IV
41	IV
30	IV
43	IV
37	IV
26	I
25	I
38	I
24	I
27	I
32	I
25	I
27	I
25	I
27	I

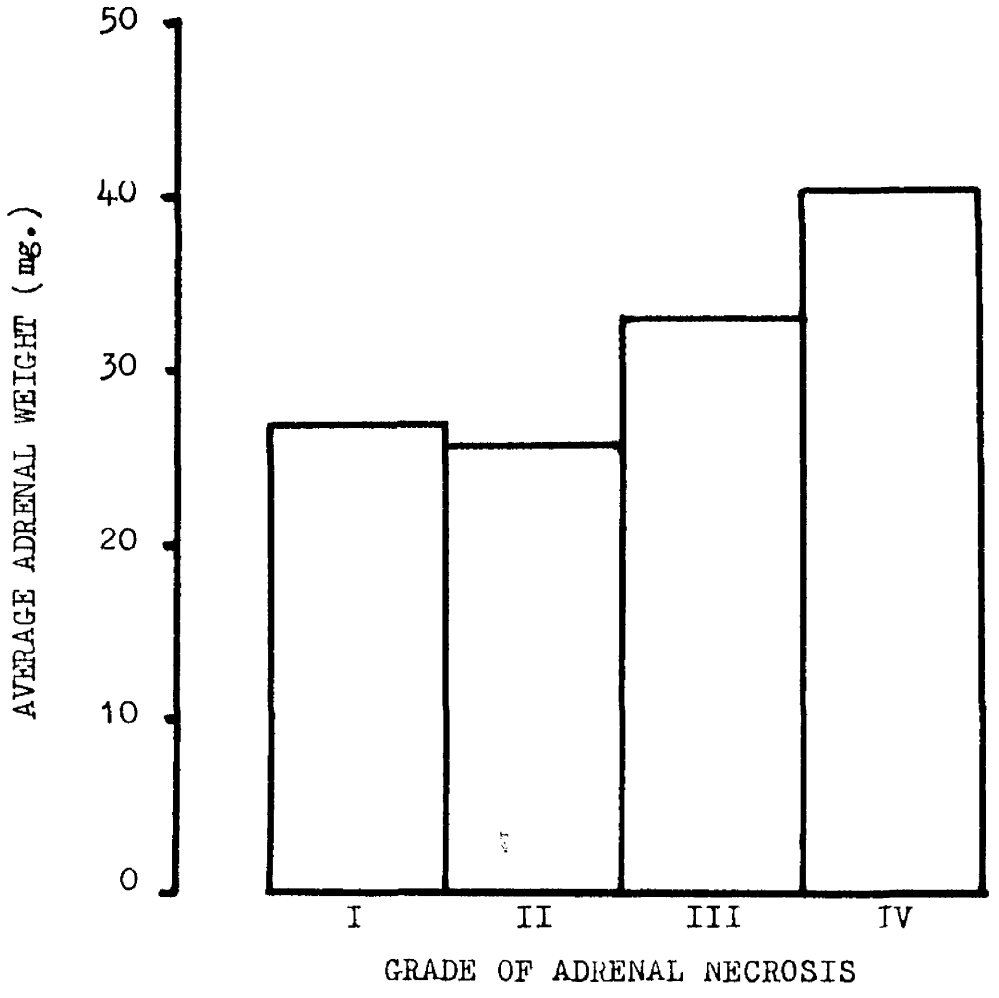
TABLE 2.

The Effect of Adrenal Necrosis on Average Adrenal Weights.

Grade of Adrenal Necrosis	Average Adrenal Weight mg.	
I	26.8	26.3
II	25.8	
III	33	36.7
IV	40.4	

FIGURE 1.

THE RELATIONSHIP BETWEEN THE GRADE OF ADRENAL NECROSIS AND THE WEIGHT OF THE ADRENAL GLAND.


50
40
30
20
10
0
AVERAGE ADRENAL WEIGHT (mg.)
I
II
III
IV
GRADE OF ADRENAL NECROSIS

RESULTS.

1. The Effect of Partial Hepatectomy on the Adrenolytic Action of DMBA.

In this experiment, groups of animals were subjected to partial hepatectomy or sham hepatectomy; others were left intact. Animals in these groups were treated 24 hours post-operatively with either 0.6 ml. of the DMBA - containing emulsion (i.e. 3 mg. DMBA) or 0.6 ml. of the control emulsion intravenously. The survivors were killed three days after the administration of the DMBA and the damage to their adrenal glands assessed.

The incidence of massive adrenal necrosis is shown in Table 3. A rat which had undergone partial hepatectomy and died on the day following DMBA treatment is excluded from the results. It is apparent that partial hepatectomy has protected the animals against the adrenolytic effect of DMBA. The difference in the incidence of massive adrenal necrosis between animals subjected to partial hepatectomy and sham hepatectomy is highly significant ($P. < 0.005$). Animals which had not undergone operation produced a 7/10 incidence of massive necrosis as would be expected with a 3 mg. dose of DMBA. Sham hepatectomy, therefore, appears to render rats more susceptible to adrenal damage from DMBA.

TABLE 3.

The Effect of Partial Hepatectomy on the Adrenolytic Action of DMBA.

Operation	Treatment	No. of rats	Massive Adrenal Necrosis
No operation	Control emulsion	10	0/10
No operation	DMBA	10	7/10
Sham hepatectomy	Control emulsion	10	0/10
Sham hepatectomy	DMBA	10	10/10
Partial hepatectomy	Control emulsion	10	0/10
Partial hepatectomy	DMBA	20	6/19

2. The Effect of Liver Damage Inflicted after DMBA Administration on the Incidence of Adrenal Necrosis.

Liver damage was produced either by carrying out partial hepatectomy or by treatment with carbon tetrachloride. The carbon tetrachloride was made up of a 50% v/v solution in olive oil and 0.3 ml. of this solution was administered by intraperitoneal injection. The operations or injections were performed at times ranging from 0 to 24 hours after the intravenous injection of 3 mg. DMBA.

Control groups of rats were subjected to sham hepatectomy or received 0.3 ml. of olive oil by intraperitoneal injection.

The animals were killed 3 days after receiving the DMBA and the adrenal damage assessed.

The results obtained are shown on Table 4 and Figure 2. It was found that:-

1. Carbon tetrachloride did not act as a protector if given after DMBA.
2. Both carbon tetrachloride and partial hepatectomy carried out up to 2 hours after DMBA increased the incidence of adrenal necrosis.
3. Partial hepatectomy performed more than 6 hours after DMBA did have some protective effect.

TABLE 4.

The Effect of Liver Damage Inflicted after DMBA (3 mg. I.V.) Administration on the Incidence of Adrenal Necrosis.

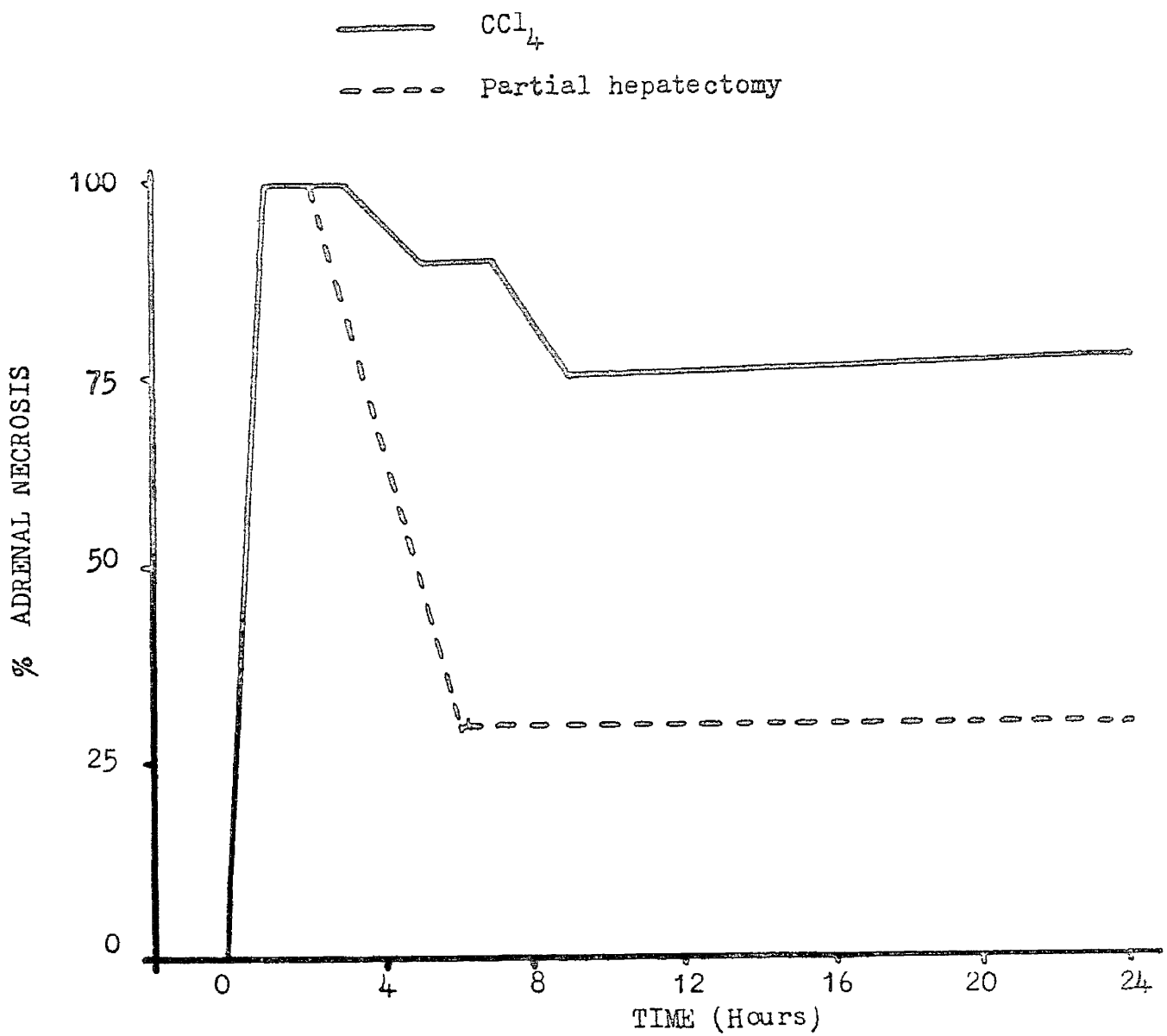
Treatment	Time after DMBA (hours)	No. of rats	Massive Adrenal Necrosis
*Olive oil	9	10	5/9
Olive oil	5	10	8/10
**CCl_4	0	10	0/8
CCl_4	1	10	10/10
CCl_4	3	10	10/10
CCl_4	5	10	9/10
CCl_4	7	10	9/10
CCl_4	9	10	6/8
CCl_4	24	10	7/9
Sham hepatectomy	6	10	6/10
Partial hepatectomy	2	10	7/7
Partial hepatectomy	6	10	3/10
Partial hepatectomy	24	10	3/10

*Olive oil = dose 0.3 ml. by intraperitoneal injection.

**CCl_4 = dose 0.3 ml. of 50% solution in olive oil by intraperitoneal injection.

FIGURE 2.

THE EFFECT OF LIVER DAMAGE WHEN INFLICTED AFTER DMBA ADMINISTRATION (3 mg. I.V.) ON THE INCIDENCE OF ADRENAL NECROSIS.


CCl_4
Partial hepatectomy
100
75
50
25
0
% ADRENAL NECROSIS
0
4
8
12
16
20
24
TIME (Hours)

3. The Effect of Thioacetamide on the Adrenolytic Action of DMBA.

The hepatotoxic agent thioacetamide was administered to rats in varying doses and at various time intervals in relationship to a challenge dose of 3 mg. DMBA (I.V.). The thioacetamide was made up as a 2% aqueous solution and given by intraperitoneal injection. Adrenal damage was assessed three days after DMBA treatment.

As shown in Table 5, a wide range of doses of thioacetamide failed to provide protection when administered 24 hours after DMBA. With the 3 mg. DMBA used a 70% incidence of massive necrosis would be expected whereas a 100% incidence of necrosis resulted.

When a standard dose of thioacetamide (200 mg./Kg. body weight) was administered at various times from 24 hours before, to 24 hours after, the DMBA treatment, protection of the adrenal glands was demonstrated as recorded in Table 6. The percentage incidence of adrenal necrosis illustrated by Figure 3 shows that:-

1. Thioacetamide provided mild protection if given 6 hours after DMBA.
2. The adrenolytic effect of DMBA was enhanced if thioacetamide was given 3 hours before or 12 hours after DMBA.

TABLE 5

The Effect of Thioacetamide on the Adrenolytic Action of DMBA (3 mg. I.V.) when given 24 hours after DMBA.

Thioacetamide mg./Kg. body weight. I.P.	No. of rats.	Massive Adrenal Necrosis.
10	5	5/5
50	5	5/5
100	5	5/5
200	5	5/5
300	5	5/5
400	5	4/5
500	5	5/5

TABLE 6.

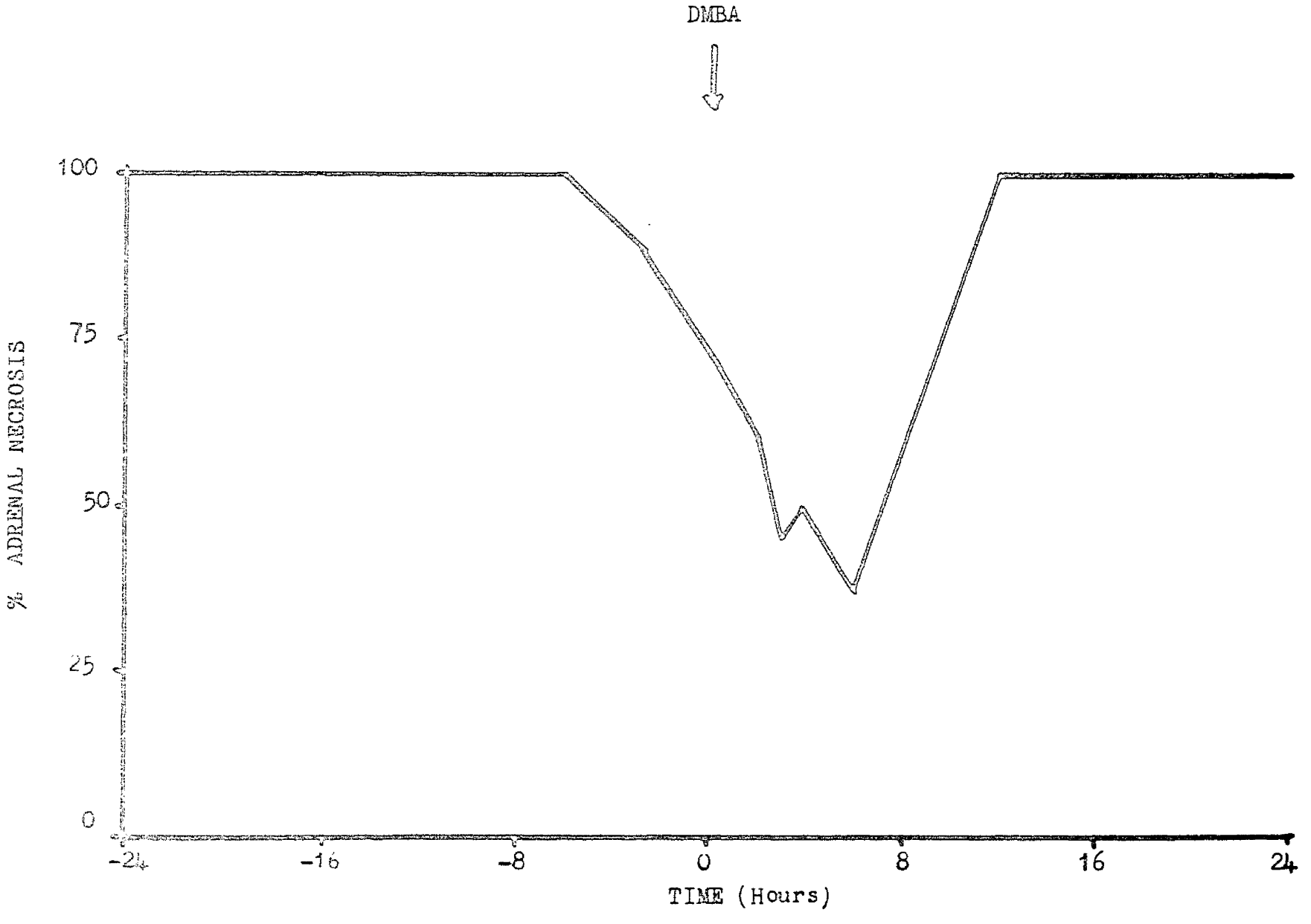
The Effect of Thioacetamide on the Adrenolytic Action of DMBA (3 mg. I.V.) when Administered at Various Times in Relationship to the DMBA.

(Dosage of Thioacetamide = 200 mg./Kg. body weight by intraperitoneal injection.)

Time Related to DMBA(hours)	No. of Rats	Massive Adrenal Necrosis
-24	5	5/5
-12	5	5/5
-6	10	10/10
-3	10	9/10
0	15	11/15
+2	10	6/10
+3	11	5/11
+4	10	5/10
+6	11	4/11
+12	5	5/5
+24	5	5/5

FIGURE 3.

THE EFFECT OF THIOACETAMIDE ON THE ADRENOLYTIC ACTION OF DMBA (3 mg. I.V.)


DMBA
100
75
50
25
0
% ADRENAL NECROSIS
-24
-16
-8
0
8
16
24
TIME (Hours)

4. The Effect of Liver Damage on Adrenolytic Protection Induced by Pretreatment with DMBA.

Pretreatment with a small dose of DMBA will protect rats from adrenal damage induced by a challenge dose of DMBA. Since the adrenolytic effect of DMBA can be modified by interference with liver function, it was considered possible that the liver might also play a part in the protection induced by the pretreatment with DMBA.

All animals received a challenge dose of 5 mgm. DMBA intravenously. Pretreatment with either 1 mg. DMBA intravenously or an equivalent volume of control emulsion was carried out 24 hours prior to the challenge dose of DMBA. Liver damage was inflicted with carbon tetrachloride which was administered as a 50% solution in olive oil. Different groups of rats received either 0.1 ml. or 0.2 ml. of the solution intraperitoneally; these injections were carried out either two hours before or simultaneously with the injection of the pretreatment dose of DMBA. A control group received an intraperitoneal injection of 0.2 ml. olive oil at the same time as the DMBA challenge. Adrenal damage was assessed three days later.

The following conclusions were drawn from the results shown in Table 7:-

1. The protection afforded by pretreatment with 1 mg. DMBA was confirmed.

2. The protection afforded by pretreatment with carbon tetrachloride was confirmed.

3. The larger dose of carbon tetrachloride provided a greater degree of protection.

4. Treatment with carbon tetrachloride reduced the degree of protection induced by a preliminary dose of DMBA.

5. The larger dose of carbon tetrachloride decreased the protection provided by pretreatment with DMBA.

6. Treatment with carbon tetrachloride either simultaneously with or two hours before the pretreatment dose of DMBA, did not influence the degree of protection.

The Effect of Liver Damage on Adrenolytic Protection Induced by Pretreatment with DMBA.

Pretreatment DMBA 1 mg. I.V. or Emulsion	Carbon Tetrachloride ml. I.P. of 50% solution	Time interval between Pretreatment and Carbon Tetrachloride Hours	No. of Rats	Massive Adrenal Necrosis	
DMBA	Olive oil	0	5	1/5	1/5
DMBA	0.1	2	5	4/5	8/10
DMBA	0.1	0	5	4/5	
DMBA	0.2	2	5	2/5	4/8
DMBA	0.2	0	5	2/3	
Emulsion	0.1	2	5	5/5	9/10
Emulsion	0.1	0	5	4/5	
Emulsion	0.2	2	5	3/4	6/8
Emulsion	0.2	0	5	3/4	

5. The Effect of dl-Ethionine on the Adrenolytic Action of DMBA.

With the demonstration that various forms of liver damage would protect rats against the adrenolytic action of DMBA the effect of treatment with dl-ethionine was investigated as this substance is known to inhibit protein synthesis as well as being hepatotoxic.

The dl-ethionine was dissolved in normal saline to produce a solution containing 25 mg. dl-ethionine per ml. Glycine, the simplest amino acid, was selected at random to act as a control; it was prepared as a solution of similar concentration. The amino acids were administered by intraperitoneal injection in a dosage of 1 mg. per g. body weight to rats which had been starved overnight. The treatment with the amino acids was carried out at various time intervals in relationship to the administration of a challenge dose of 3 mg. DMBA intravenously. Adrenal damage was assessed as usual. The results are shown in Table 8 and Figure 4.

These experiments suggested that dl-ethionine provided mild protection when administered three days before a challenge dose of DMBA. The striking result, however, was the good degree of protection afforded by glycine. This had not been expected.

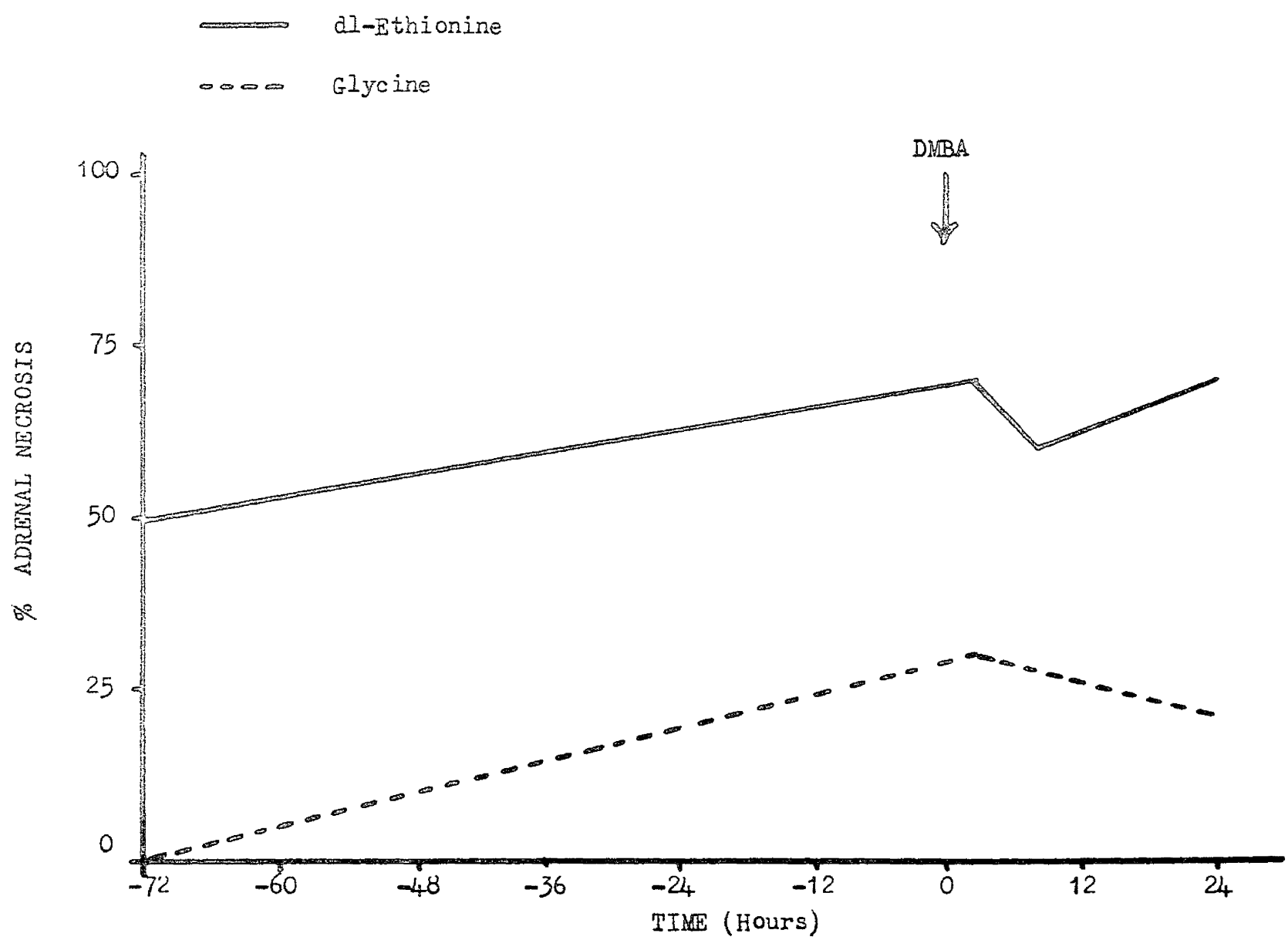
TABLE 8

The Effect of dl-Ethionine on the Adrenolytic Action of DMBA.

Amino Acid	Time related to DMBA (Hours)	No. of Rats	Massive Adrenal Necrosis
Ethionine	-72	10	5/10
Ethionine	+2	10	7/10
Ethionine	+8	10	6/10
Ethionine	+24	10	7/10
Glycine	-72	10	0/10
Glycine	+2	10	3/10
Glycine	+24	9	2/9

FIGURE 4.

THE EFFECT OF dl-ETHIONINE AND GLYCINE ON THE ADRENOLYTIC ACTION OF DMBA (3 mg. I.V.)


dl-Ethionine
Glycine
DMBA
100
75
50
25
0
% ADRENAL NECROSIS
-72
-60
-48
-36
-24
-12
0
12
24
TIME (Hours)

6. The Effect of Nembutal on the Adrenolytic Action of DMBA.

The phenothiazine derivative Nembutal was tested for its ability to protect the adrenals. A stock solution containing 60 mg. per ml. was diluted tenfold. A dose of 2.5 mg. per 100 g. body weight was administered by intraperitoneal injection. The results obtained according to various dosage schedules are shown in Tables 9 and 10. Figures 5 and 6 illustrate that Nembutal provides weak protection against 3 mg. DMBA but that such an effect is masked by 5 mg. DMBA. It is apparent that the protection established by Nembutal is present 48 hours after its administration.

The Effect of a Single Pretreatment Dose of Nembutal on the Adrenolytic Effect of DMBA.

DMBA mg.	Nembutal 2.5 mg./100 g. body weight. Days before DMBA	No. of Rats	Massive Adrenal Necrosis
5	-5	5	5/5
5	-3	5	5/5
5	-1	5	5/5

TABLE 10.

The Effect of Repeated Daily Doses of Nembutal on the Adrenolytic Effect of DMBA.

DMBA mg.	Nembutal 2.5 mg./100 g. body weight I.P. No. of Daily Injections Prior to DMBA.	Massive Adrenal Necrosis
5	5	5/5
5	4	5/5
5	3	4/5
5	2	5/5
5	1	5/5
3	5	2/6
3	4	1/6
3	3	1/6
3	2	1/6
3	1	4/6

FIGURE 5.

THE EFFECT OF NEMBUTAL (PENTOBARBITONE SODIUM) ON THE ADRENOLYTIC ACTION OF DMBA (5 mg. I.V.)

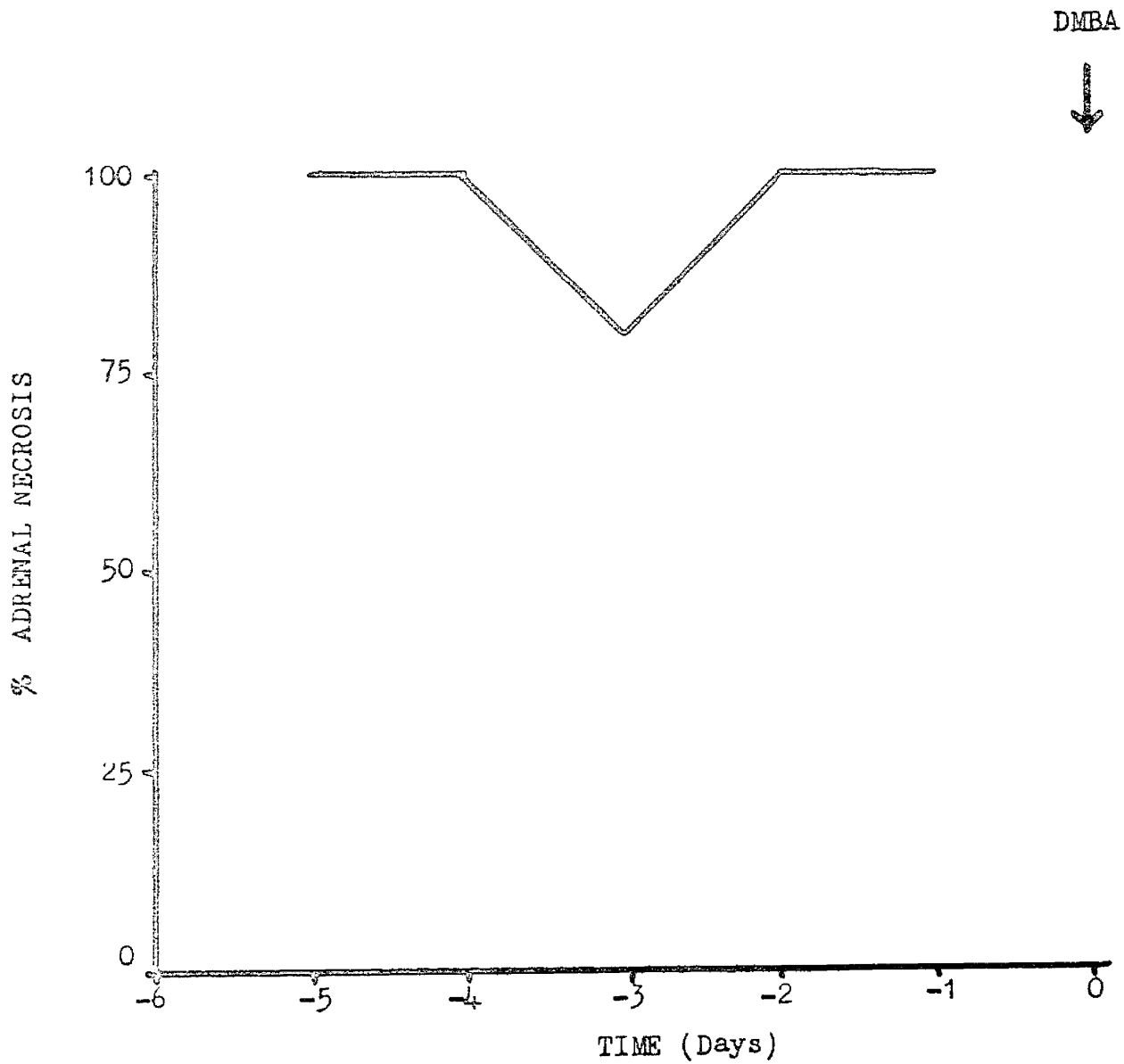
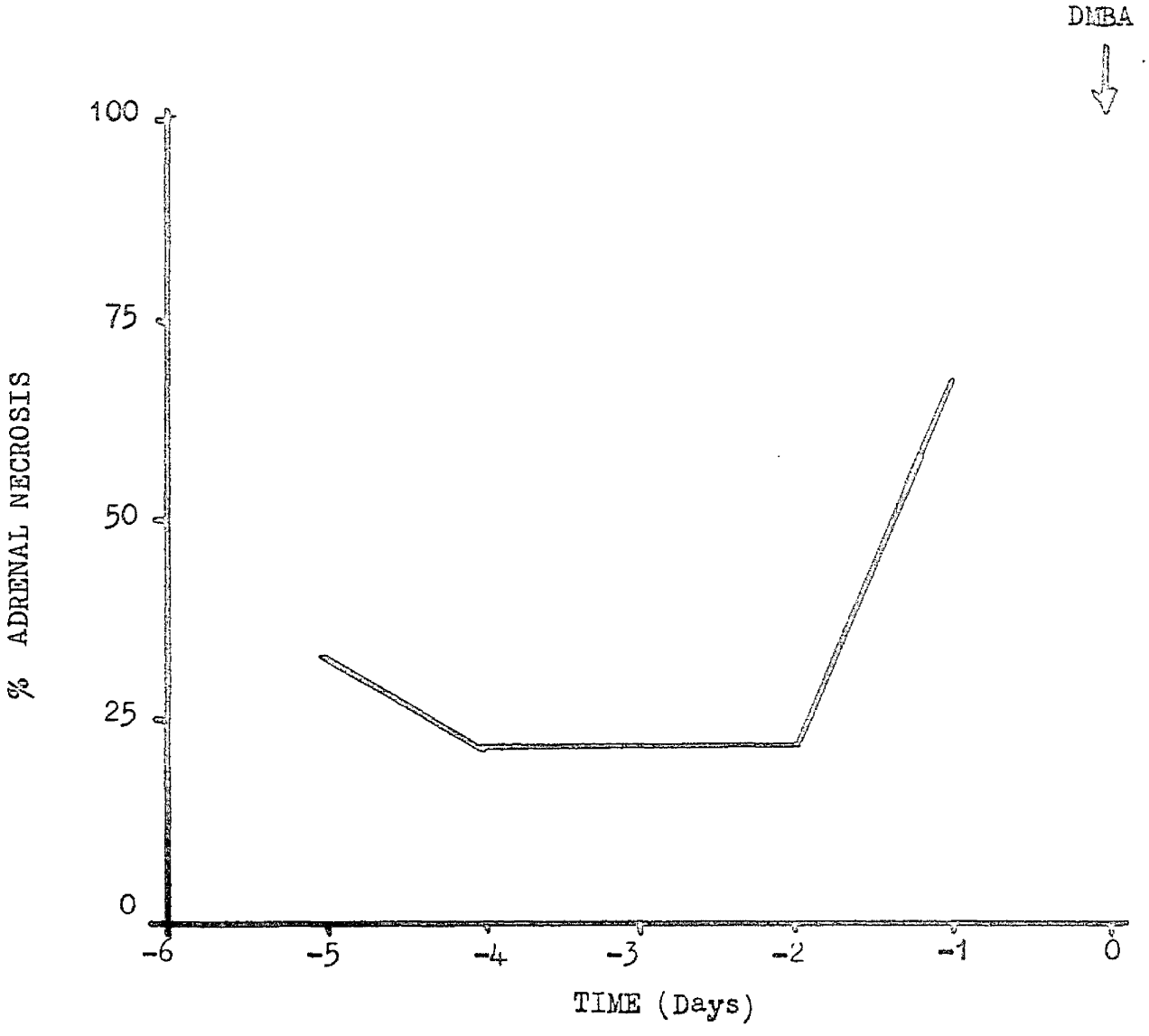

DMBA
100
75
50
25
0
% ADRENAL NECROSIS
-6
-5
-4
-3
-2
-1
0
TIME (Days)

FIGURE 6.

THE EFFECT OF NEMBUTAL (PENTOBARBITONE SODIUM) ON THE ADRENOLYTIC ACTION OF DMBA (3 mg. I.V.)


DMBA
100
75
50
25
0
% ADRENAL NECROSIS
-6
-5
-4
-3
-2
-1
0
TIME (Days)

DISCUSSION.

Based on the hypothesis that liver metabolism plays an important role in the adrenolytic action of DMBA, a series of experiments has been carried out to investigate the effect of liver damage on DMBA induced adrenal necrosis. Although carbon tetrachloride, which is a well known hepatotoxic agent had been shown to provide protection (Wheatley, 1965 - personal communication), it was felt necessary to test the effects of other forms of liver damage. It has now been shown that partial hepatectomy and thioacetamide poisoning will also provide protection, although neither gave such a solid protection as did carbon tetrachloride.

Carbon tetrachloride produces a fatty liver and it was considered feasible that its protective action might be explained by the sequestration of the lipid-soluble DMBA in the fat globules present in the liver. However, such a mechanism could not account for the protection afforded by partial hepatectomy or thioacetamide poisoning. The common factor of liver damage appears to be the essential cause of adrenal protection in these cases.

When the time relationship between protective measures and DMBA treatment are studied differences are noted between the various methods. Thus, both carbon tetrachloride and partial hepatectomy give good protection when treatment is

carried out 24 hours before DMBA administration, whereas thioacetamide exerts its main protective influence when given 6 hours after DMBA. If adrenal protection is related to the degree of liver function then these findings suggest that carbon tetrachloride, partial hepatectomy and thioacetamide produce maximal liver damage at different times after treatment. Moreover, as the degree of protection also varies with each agent it appears that the amount of liver damage that they infliot also differ. That the degree of liver damage is important is demonstrated by the fact that whereas 0.07 ml. 50% carbon tetrachloride in oil does not provide adrenal protection (Tanaka and Dao, 1965), 0.3 ml. of 50% carbon tetrachloride in olive oil will do so (Wheatley, Kernohan and Currie, 1966).

It was observed that partial hepatectomy after DMBA would still provide protection unlike post treatment with carbon tetrachloride (Figure 2). Such an effect could be explained by the mechanical removal of DMBA immobilised in the liver.

The protection afforded by pretreatment with hydrocarbons including DMBA itself, has been ascribed to the induction of those enzyme systems responsible for the inactivation of the foreign material (Wattenberg, and Leong, 1965). Carbon tetrachloride treatment has now been shown to inhibit the production of protection by a pretreatment dose of DMBA (Table 7). Such a finding supports the hypothesis that liver

damage exerts its influence by interfering with enzyme systems involved in the metabolism of DMBA.

If enzyme induction is of importance then the blocking of protein synthesis by dl-ethionine would be expected to influence DMBA metabolism. Such an effect has been noted, (Huggins and Fukunishi, 1964) and these present investigations have suggested that dl-ethionine treatment 3 days before DMBA would provide adrenal protection of a mild degree (Table 8). The length of time between the dl-ethionine and DMBA treatments necessary to allow the establishment of protection was noted and has been further investigated (Wheatley, 1969). The most striking feature of this initial study however, was the fact that glycine, which had been selected at random to serve as a 'control' amino acid, produced a much greater degree of protection than did dl-ethionine which was the subject of the investigation (Table 8). It was considered possible that glycine exerted its effect by virtue of its metabolism to ammonia which then produced liver damage. It has now been suggested that dl-ethionine acts as a protector by delaying the uptake of DMBA by the liver (Wheatley, 1969).

Pretreatment with phenobarbital provides protection (Huggins and Fukunishi, 1964) and since it also induces an increase in liver benzpyrene hydroxylase (Wattenburg and Leong, 1965) it would appear to act by stimulating the enzyme systems involved in DMBA inactivation. In particular it

increases the rate of drug hydroxylation (Orrinis and Ernster, 1964). The chemically related substance Nembutal has been tested for its protective ability and has been found to be a protector of moderate degree (Figure 6). It is of interest that the barbiturates which induce a smaller amount of benspyrene hydroxylase than phenothiazine (Wattenberg and Leong, 1965) are found to be less efficient as adrenal protectors. Such a correlation suggests that the ability to bring about enzyme induction is related to the ability to afford adrenal protection against DMBA.

The metabolism of the polycyclic hydrocarbons in the rat has been studied by other workers. Whereas benz(a)anthracene is metabolised by oxidation by selected centres of unsaturation. (Boyland and Sims, 1964; Boyland, Kimura and Sims, 1964) DMBA undergoes oxidation of its methyl groups (Boyland and Sims, 1965) to form principally 7-hydroxymethyl-12-methylbenz(a) anthracene (7-OHM-12-MBA) and 12-hydroxymethyl-7-methylbenz(a) anthracene (12-OHM-7-MBA). When these metabolites were tested for adrenolytic properties (Boyland, Sims and Huggins, 1965) it was found that 7-OHM-12-MBA was more active than DMBA (5 mg. 7-OHM-12-MBA was equivalent to 30 mg. DMBA when administered orally), but 12-OHM-7-MBA was inactive. The related compound 7 hydroxymethylbenz(a)anthracene was also inactive indicating that a methyl group in the 12 position was essential for this property.

Further studies have revealed that treatment such as carbon tetrachloride or partial hepatectomy which produce liver damage and protect the adrenals DMBA will not protect against 7-OHM-12-MBA (Wheatley, Hamilton, Currie, Boyland and Sims, 1966). These findings support the hypothesis that the adrenolytic effect of DMBA is due to its conversion to the biologically active 7-OHM-12-MBA by the liver.

The resistance of immature rats to the adrenolytic effect of DMBA has been ascribed to the inability of their adrenals to synthesize corticosterone. Hypophysectomy and treatment with Metapirone which depress adrenal function, likewise protect against DMBA. Metapirone has also been reported to protect against 7-OHM-12-MBA (Wheatley, Hamilton, Currie, Boyland and Sims, 1966) which would be in keeping with the hypothesis that only a gland actively synthesizing corticosterone or related steroids is susceptible to damage.

When the effect of dl-ethionine and glycine was investigated it was apparent that these techniques did not give protection of adrenals against 7-OHM-12-MBA (Wheatley, 1969).

SKF525A which inhibits the detoxification of various drugs by the microsonal enzymes in mammalian liver has been shown to protect the adrenals against both DMBA and 7-OHM-12-MBA (Wheatley, 1968i). This finding has raised the possibility that either 7-OHM-12-MBA is metabolised to an active form or else a new form of protective mechanism exits.

methionine did not abolish the SKF 525A - protection against DMBA but did partially abolish the protection against 7-OH-12-MBA.

Various inhibitors of adrenal corticosteroid synthesis such as SU 4885 (metyrapone), SU 9055 and SU 10603, have been found to protect against both DMBA and 7-OH-12-MBA. This protection is abolished by treatment with di-ethionine (Huntley, 1966b). It was, therefore, concluded that this form of protection was due to the stimulation of drug metabolising enzymes in the liver and did not depend on the depression of adreno-corticosteroid synthesis. Ellipton which suppresses both ovarian and corticosterone production and SU 9074 which inhibits 11β hydroxylation were not protectors. Hence there did not appear to be a correlation between the ability to act as a protector and to inhibit steroid synthesis by the adrenals.

More detailed investigation of the metabolism of DMBA (Boyland and Sims, 1967) suggests that the hydroxymethyl derivatives undergo both conversion to carboxylic acids and ring hydroxylation. Phenobarbitone is reported as increasing the rate of metabolism of methyl and hydroxymethyl groups whilst metyrapone increases the rate of hydroxylation mainly of the methyl groups but to a lesser extent of the ring systems. Study of the biochemical effect of pretreatment with DMBA suggests that this shifts the metabolism from hydroxylation of the side chains to the ring to produce biologically inactive compounds (Jellinck and Boyd, 1966).

The enzymes involved in DMBA metabolism appear to be age-dependent since there is an increased rate of metabolism of DMBA in rats aged 25 days (Sims and Grover, 1967). It has been suggested that this is due to induction of these enzymes by the dietary components first ingested during weaning.

Because of its structural similarity to the steroids it has been postulated that DMBA produces adrenal damage by an irreversible binding to sites within those cells responsible for steroid synthesis, so interfering with the electron transport system (Wong and Warner, 1964). The possibility has been raised that compounds which protect by the inhibition of protein synthesis might exert their effect by acting directly on the adrenals (Dao and Varela, 1966). It has, however, been shown that treatments which alter the metabolism of DMBA by the liver and result in protection against the adrenolytic effect do not affect the metabolism of DMBA by the adrenal glands themselves (Jellinck and Goudy, 1967).

The results of the experiments in the present investigation have demonstrated that the liver does control the adrenolytic effect of DMBA but it has been noted that procedures which have been used as controls such as sham hepatectomy and unilateral nephrectomy (Wheatley, Kernohan and Currie, 1966) or treatments with agents such as thioacetamide and carbon tetrachloride outwith the critical times for protection will enhance the incidence of adrenal necrosis (Figure 2 and

Figure 3). It seems likely that any treatment that induces stress results in an increased functional activity of the adrenal cortex and that cells with an increased rate of synthesis of corticosterone are more susceptible to damage by DMBA.

Although the functional activity of both liver and adrenal is important for the development of adrenal necrosis in response to DMBA, it is accepted that other factors must be involved. The fact that the effect is species-specific to the rat supports such a contention. It may well be that the adrenal lysosomes or vascular bed of the rat show a peculiar sensitivity to 7-OHM-12-MBA (Jellinck, Coles and Garland, 1967).

PART II

PROTECTION AGAINST THE CARCINOGENIC EFFECT OF DMBA.

REVIEW OF THE CARCINOGENIC EFFECT OF DMBA.

With the discovery that the mammary tumour induced in rats by the oral administration of 3-methylcholanthrene were hormone dependent (Huggins, 1958; Huggins, 1959; Huggins, Briziarelli and Sutton,1959) an important experimental model became available. Further studies revealed that all female Sprague-Dawley rats aged 50 - 65 days wouldddevelop mammary tumours following a single dose of 20 mgm. DMBA orally or 5 mgm. DMBA intravenously (Huggins, Morii and Grand, 1961). It was concluded (Huggins and Yang, 1962) that the following factors were of importance for the induction of mammary tumours:-

1. Nature of the hydrocarbon.
2. Dose of the hydrocarbon.
3. Species of the experimental subject.
4. Strain of the experimental subject.
5. Age of the experimental subject.
6. Hormonal status of the experimental subject.

A strong carcinogenic stimulus was later shown to be able to nullify the inherited strain differences in susceptibility (Sydnor, Butenandt, Brillantes and Huggins, 1962).

The histological appearances of the mammary tumours induced by 3-methylcholanthrene in intact Sprague-Dawley female rats were studied (Huggins, Briziarelli and Sutton, 1959) and classified as follows:-

Carcinoma	678
Fibrosarcoma	2
Benign	0

Macroscopically the carcinomas were white and soft; haemorrhage and necrosis were frequently encountered in parts of the tumour. Large tumours often developed ulceration and although infiltration of adjacent muscles was noted distant metastases were not observed. Histologically the tumours consisted of acini lined with many layers of epithelial cells arranged to form gland-like structures with papillary projections. The lumina were filled with an eosinophilic material.

It was pointed out that the histological appearances of tumours induced with DMBA resembled human mammary cancer although the latter is usually more anaplastic (Young, 1961). There was considerable histological variation within the same tumour, but no differences could be detected between the hormone sensitive and insensitive types of tumour (Young, Cowan, and Sutherland, 1963).

The natural history of these tumours has been described (Young and Cowan, 1963). It was found that:-

(a) Some tumours could be detected by palpation at 31 days although most appeared between 50 - 100 days.

(b) Three types of tumour could be distinguished by their growth characteristics, namely,

(i) tumours continuing to grow steadily (25%)

(ii) tumours whose growth stopped and remained the same size for months (50%)

(iii) tumours which stopped growing and then regressed (25%).

The tumours which became static or regressed were noted to have a slower growth rate initially.

(c) The histological appearances of spontaneously regressing tumours resembled those found in growing tumours.

Further histological studies have shown that tumours are multifocal in origin (Middleton, 1965).

Although the individual tumours show a variation in histological appearance it has now been demonstrated that there is a correlation between the tumour's histology and its biological behaviour (Stevens, Stevens and Currie, 1965). Rapidly growing tumours have the appearance of anaplastic adenocarcinomas, whilst the regressing tumours appear to be benign adenomas with an acinor or microcystic structure. A similar correlation has been reported by others (Archer and Orlando, 1968).

The effects of various manipulations of the hormonal status of the host have been described by many workers and the following factors have been found to influence tumour induction or tumour growth:-

Oestrogens - (Huggins and Yang, 1962; Huggins, Moon and Morii, 1962; Dao, 1962; Huggins, 1963ii; Shimkin, Gropper, Thatcher and Gruenstein, 1967; Jabara, 1967; Heimann, Heuson and Coune, 1968).

Progesterone - (Huggins and Yang, 1962; Huggins, Moon and Morii, 1962; Huggins, 1963ii).

Pituitary and hypothalamic hormones - (Daniel and Pritchard, 1967; Clemens, Welsch and Meites, 1968).

Thyroid hormone - (Helfenstein, Young and Currie, 1962).

Androgens - (Young, Baker and Helfenstein, 1965; Heise and Görlich, 1966).

Relaxin - (Plunkett and Gammal, 1967).

Pregnancy, lactation and suckling - (Huggins and Young, 1962; McCormick and Moon, 1965; McCormick and Moon, 1967i; McCormick and Moon, 1967ii).

That environmental factors are also of importance is apparent from the reported increased incidence of tumours in rats kept in a heated atmosphere (Young, 1968).

There is some evidence that the more anaplastic types of tumour tend to develop in rats which have heavier ovaries and lighter pituitary glands than in those animals bearing differentiated tumours of a fibroadenomatous pattern (Hamilton and Sneddon, 1968), suggesting that the hormonal environment influences the type of tumour induced.

Investigation of the changes occuring in the tumours during regression induced by oophorectomy has been carried out. There is no dramatic change in the levels of nucleic acids and proteins within 48 hours of operation (Stevens, 1966). Electronmicroscopy has not revealed any difference between endocrine-dependent and endocrine-independent growing tumours or between spontaneously regressing tumours and those that regress after oophorectomy, but it has established that atrophy is the dominant visible cellular change associated with regression (Scott, Christian and Currie, 1967).

Following the demonstration that the adrenolytic effect of DMBA could be prevented by Metopirone an attempt was made to prevent the carcinogenic effect of DMBA by the same means (Helfenstein and Young, 1963). It was found to reduce the total yield of mammary cancers but did not materially affect the induction period of the tumours or the proportion of rats bearing tumours. However, when hydrocarbons which acted as adrenal protectors were fed to rats the induction of mammary tumours by DMBA was suppressed (Huggins, Grand and Fukunishi, 1964). It has, therefore, been suggested that ability to protect the adrenals might serve as a screening test in the search for compounds to protect against carcinogenesis (Morii, 1965). The ability of hydrocarbons to act as protectors against carcinogens is believed to be due to the fact that they induce detoxifying enzymes (Wattenberg, 1966).

Having demonstrated that impairment of liver function would protect against the adrenolytic effect of DMBA it was decided to investigate what effect liver function had on the carcinogenic action of DMBA. It is known that DMBA is capable of producing a variety of tumours such as leukaemia (Fukunishi, Ford and Huggins, 1965; Huggins and Sugiyama, 1966), lung tumours (Walters and Roe, 1964; Walters, 1966), subcutaneous sarcomas (Roe, Carter and Percival, 1967), melanotic tumours (Walters, Roe and Levene, 1967), and peritoneal sarcomas (Huggins and Fukunishi, 1963). This present study, however, is limited to the production of mammary tumours in Sprague-Dawley rats.

MATERIALS AND METHODS.

Tumours were induced by the intravenous administration of DMBA emulsion. The animals and the protective measures they were subjected to were the same as described in Part I of this thesis.

The combined treatment of DMBA with the protective measure resulted in a high early mortality. After the fourth week from the time of DMBA injection the surviving animals were palpated twice weekly until the experiment was terminated at six months. During each examination the tumours were measured and the tumour index charted to produce a growth curve for each tumour as described previously (Stevens, Stevens and Currie, 1965). Necropsy was performed at the end of the experiment and all tumours were submitted to histological examination.

The rats were housed under identical conditions to avoid differences in tumour production due to variations in environmental temperature (Young, 1968).

RESULTS.

The Effect of Liver Damage on Mammary Tumours Induced with 5 mg. DMBA.

Groups of rats aged 50 days were submitted to either partial hepatectomy or intraperitoneal injection of 0.3 ml. of 50% carbon tetrachloride in olive oil to produce liver damage. As a control series, other rats received an intraperitoneal injection of 0.3 ml. of olive oil. Each rat was given 5 mg. of DMBA intravenously 24 hours after these treatments.

The incidence of mammary tumours induced is recorded in Table 11 and Figure 7. There was no significant difference in either the percentage of animals developing tumours or the number of tumours developing per rat in the three experimental groups. The biological behaviour and histological appearances of the tumours were also noted to be similar in the three groups.

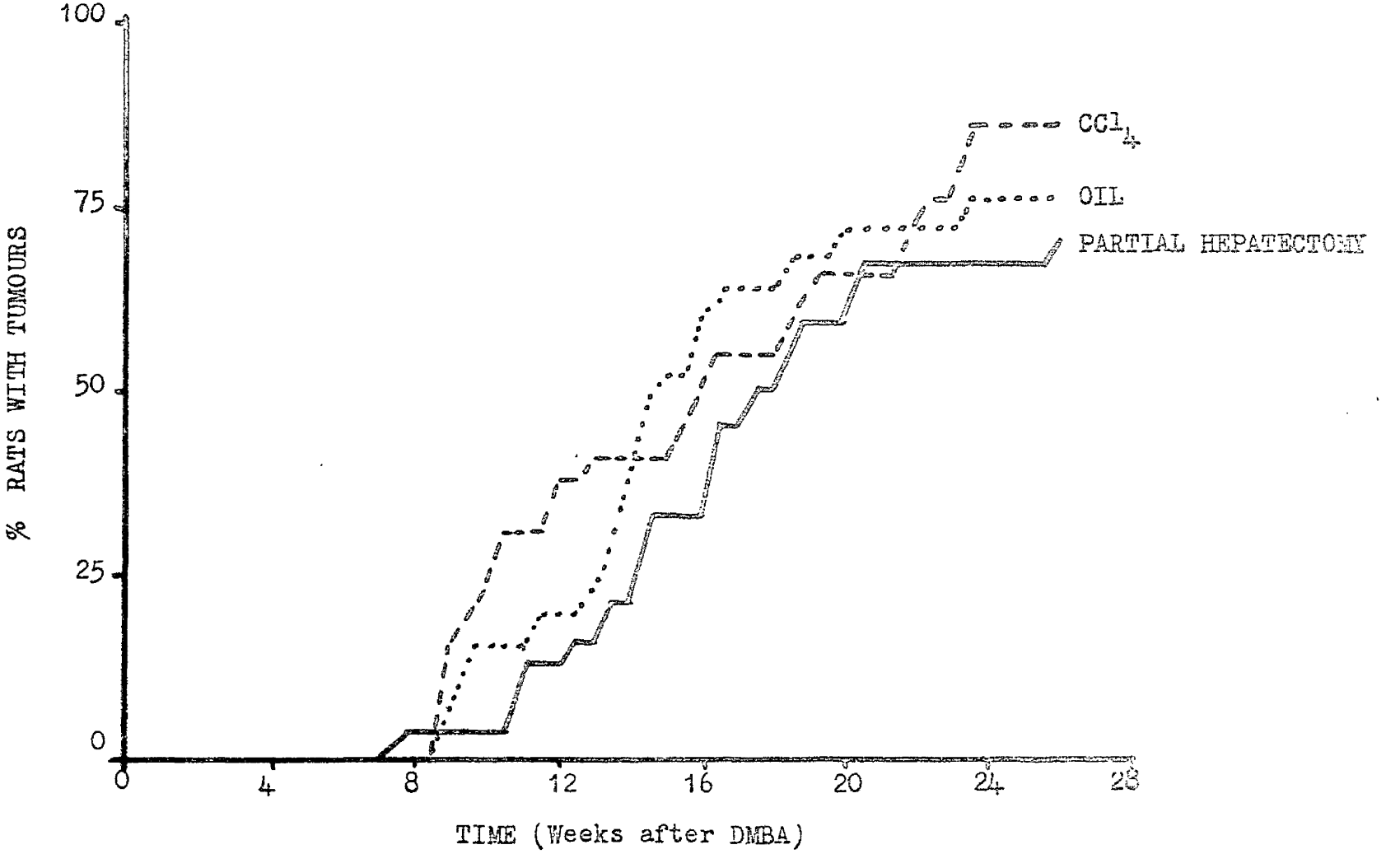
TABLE 11

Mammary Tumours in Rats Given DMBA (5 mg. I.V.) After Liver Interference.

Group	No. of Rats	No. of Rats with tumours	% of Rats with tumours	Total No. of tumours	Average No. of tumours/ tumour-bearing rat
Olive Oil	40	19/25	76	39	2.05
Carbon Tetrachloride	60	25/29	86	64	2.56
Partial Hepatectomy	43	17/24	70	36	2.12

FIGURE 7.

THE INCIDENCE OF MAMMARY TUMOURS RESULTING FROM THE ADMINISTRATION OF DMBA (5 mg. I.V.)


100
75
50
25
0
% RATS WITH TUMOURS
0
4
8
12
16
20
24
28
TIME (Weeks after DMBA)
CCl_4
OIL
PARTIAL HEPATECTOMY

The Effect of Liver Damage on Mammary Tumours Induced with 2.5 mg. DMBA.

The previous experiment was repeated using a tumour-inducing dose of 2.5 mg. DMBA intravenously administered 24 hours after the production of liver damage. An additional group of rats was subjected to sham hepatectomy to serve as an extra control series.

The results are recorded in Table 12 and Figure 8. The differences between the group as regards percentage incidence of tumours and number of tumours per rat is not regarded as significant. Once again the biological behaviour and histological pattern of the tumours did not reveal any change that could be ascribed to the protective techniques employed.

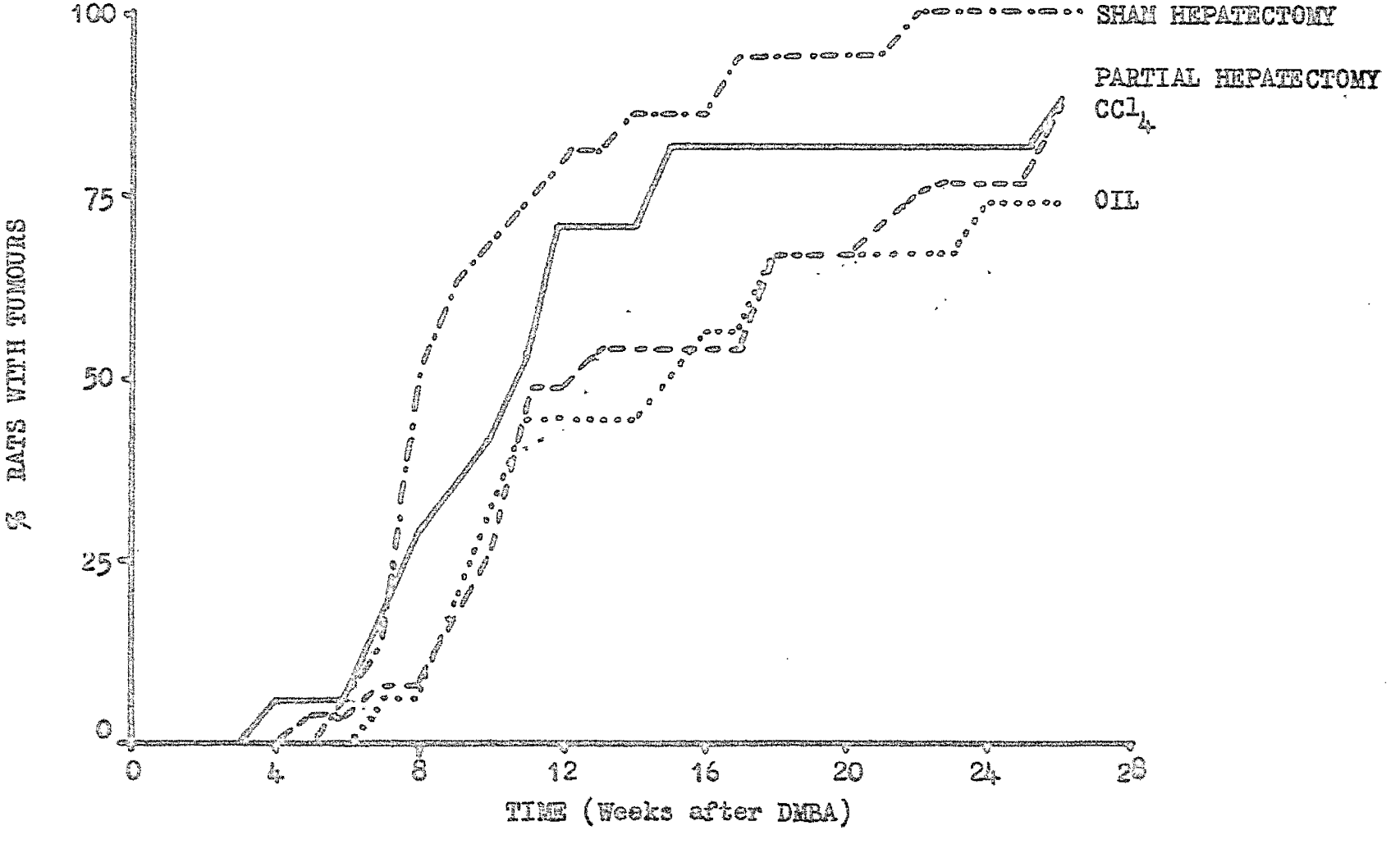
TABLE 12.

Mammary Tumours in Rats Given DMBA (2.5 mg. I.V.) After Liver Interference.

Group	No. of Rats	No. of Rats with tumours	% of Rats with tumours	Total No. of tumours	Average No. of tumours/ tumour-bearing rat
Olive Oil	20	13/18	72	45	3.24
Sham Hepatectomy	20	16/16	100	62	3.88
Carbon Tetrachloride	30	21/24	88	66	3.14
Partial Hepatectomy	30	15/17	88	66	4.4

FIGURE 8.

THE INCIDENCE OF MAMMARY TUMOURS RESULTING FROM THE ADMINISTRATION OF DMBA (2.5 mg. I.V.)


100
75
50
25
0
% RATS WITH TUMOURS
0
4
8
12
16
20
24
28
TIME (Weeks after DMBA)
SHAM HEPATECTOMY
PARTIAL HEPATECTOMY
CCl_4
OIL

DISCUSSION.

The association of a unique adrenolytic effect and a potent carcinogenic action has led previous investigators to study the relationship of these activities of DMBA. The fact that Metopirone did not effect tumour production (Helfenstein and Young, 1963) suggested that active adrenal function was not necessary for a carcinogenic effect. Since pretreatment with aromatics could suppress tumour induction (Huggins, Grand and Fukunishi, 1964) it seemed likely that an increased rate of detoxification of DMBA would prevent its carcinogenic action. When rats were pretreated with carbon tetrachloride tumour production was not impaired (Tanaka and Dao, 1965). The administration of this hepatotoxic agent would be expected to interfere with the liver enzymes responsible for DMBA metabolism. Since such interference did not influence the carcinogenic effect it supported the hypothesis that DMBA itself was the carcinogenic agent whereas its metabolites were inactive. It should be noted, however, that the dosage of carbon tetrachloride used in the experiments reported was insufficient to bring about adrenal protection.

Having established methods of liver damage which would protect the adrenals it was decided to apply these to the study of the carcinogenic effect. The initial experiment used a dose of 5 mg. of DMBA as this had been shown to be a reliable means of producing tumours (Huggins, Morii and Grand, 1961).

Liver damage which had been found to prevent adrenal necrosis did not influence tumour production (Figure 7).

It was considered possible that this apparent lack of effect might be due to the use of an overwhelming dose of carcinogen so that mild protection was not revealed, as in the case of Nembutal on DMBA-induced adrenal necrosis (Figure 5). To test this hypothesis the experiment was repeated using 2.5 mg. DMBA intravenously. Protection against tumour production could not be demonstrated even with this small dose of DMBA (Figure 8), thereby supporting the suggestion that DMBA was the true carcinogen. It was found that the control animals which could metabolise DMBA rapidly developed the same incidence of tumours as those in which liver function was impaired and so were unable to metabolise DMBA, suggesting that DMBA must initiate carcinogenesis soon after its administration. This concept of the rapid action of DMBA is substantiated by the fact that biochemical changes are detectable in the mammary glands within three days of treatment (Williams-Ashman and Huggins, 1961). It has even been shown that mammary glands transplanted to a normal host as soon as six hours after exposure to DMBA will still develop tumours (Dao, Tanaka and Gawlak, 1964). Following DMBA treatment the hydrocarbon which is localised in the mammary glands is gradually cleared, no measurable amount being left by seven days (Dao, King and Gawlak, 1968).

Investigations of the carcinogenic properties of the

metabolic derivatives of DMBA have been reported. The first of these reports (Boyland, Sims and Huggins, 1965) stated that 7-OHM-12-MBA had about the same (but not greater) activity in inducing mammary tumours as DMBA; 12-OHM-7-MBA did not induce tumours. Further study has suggested that 7-OHM-12-MBA is, in fact, a less potent carcinogen than DMBA (Pataki and Huggins, 1967). The same result has been obtained by other workers who have also shown that the dihydroxymethyl derivative is inactive (Wheatley and Inglis, 1968).

It has now been reported that treatment with a preliminary dose of polycyclic hydrocarbon designed to stimulate the metabolic enzyme systems will inhibit tumour production by DMBA whereas pretreatment with SKF 525A which inhibits drug metabolism enhances the carcinogenic activity of DMBA (Wheatley, 1968ii).

Such reports substantiate the hypothesis that DMBA is the active carcinogen as suggested previously (Kernohan, Inglis and Wheatley, 1967) and that its hydroxylated derivates are not responsible for this effect.

PART III

ASSESSMENT OF LIVER FUNCTION.

REVIEW.

Partial hepatectomy is an obvious method of inflicting liver damage. The standard operative technique has been estimated to remove 70% of the liver mass (Huggins and Anderson, 1931). However, the effect of partial hepatectomy on the functional activity of the liver was not established.

The biochemical changes following the administration of the hepatotoxic agents carbon tetrachloride and thioacetamide to rats have been investigated (Rees and Sinha, 1960) and from the values for the various liver function tests and serum enzymes studied graphs can be drawn to illustrate how these alter with the passage of time (Figures 9, 10, 11 and 12). It was apparent that the serum levels of various enzymes paralleled the changes in value of standard liver function tests. Any effect that depended upon the activity of liver enzyme system might, therefore, be used as an index on liver function.

Nembutal is metabolised by hydroxylation within the liver (Goodman and Gilman, 1955), and it acts on the central nervous system to produce narcosis. It has been proposed that measurement of the duration of narcosis might serve as an estimate of liver function (Cameron and Saram, 1939; Cameron, Cooray and De, 1948).

Investigation of the biological effects of DMBA has revealed the importance of liver metabolism in this respect. It was considered important to relate the influence of various

protective methods on the adrenolytic action of DMBA to the degree of liver damage they produce.

FIGURE 9.

THE EFFECT OF CARBON TETRACHLORIDE POISONING ON LIVER FUNCTION TESTS:

(From data published by REES and SINHA, 1960)

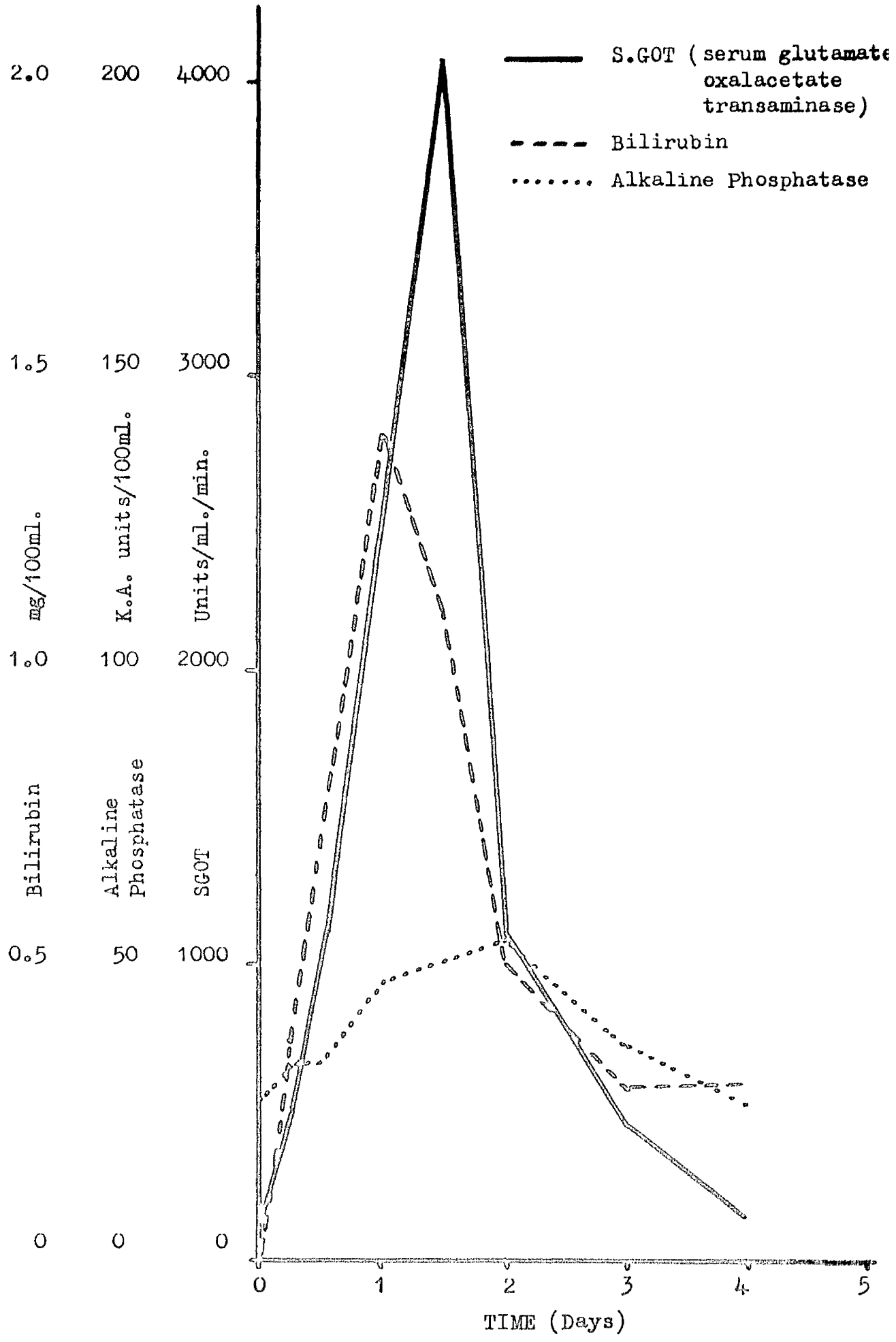

S.GOT (serum glutamate oxalacetate transaminase)
Bilirubin
Alkaline Phosphatase
2.0
200
4000
1.5
150
3000
1.0
100
2000
0.5
50
1000
0
0
0
mg/100ml.
K.A. units/100ml.
Units/ml./min.
Bilirubin
Alkaline Phosphatase
SGOT
0
1
2
3
4
5
TIME (Days)

FIGURE 10.

THE EFFECT OF CARBON TETRACHLORIDE POISONING ON BLOOD ENZYMES:

(from data published by REES and SINHA, 1960).

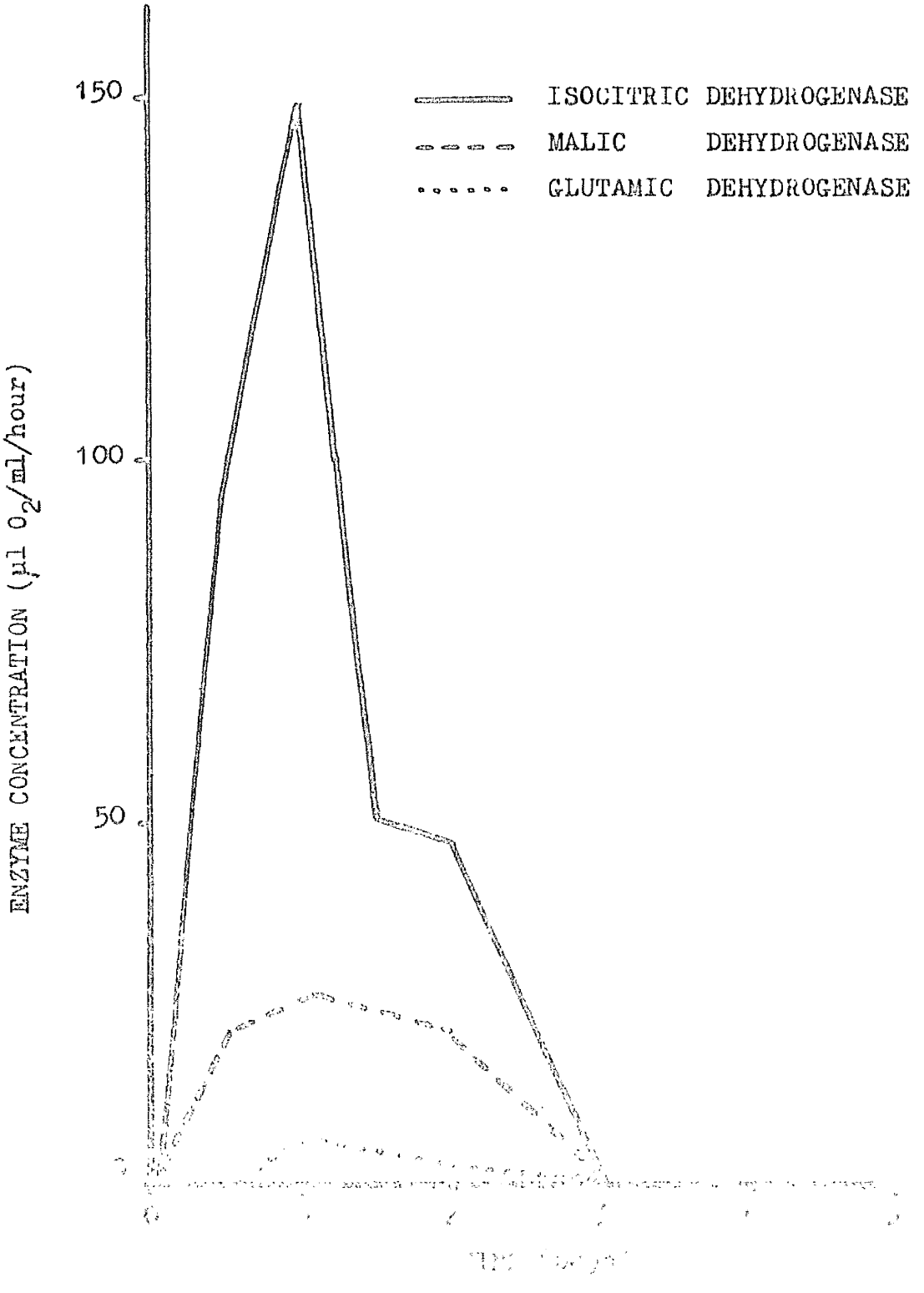

150
100
50
ENZYME CONCENTRATION (µl O_2/ml/hour)
ISOCITRIC DEHYDROGENASE
MALIC DEHYDROGENASE
GLUTAMIC DEHYDROGENASE

FIGURE 11.

THE EFFECT OF THIOACETAMIDE POISONING

ON LIVER FUNCTION TESTS:

(from data published by REES and SINHA, 1960).

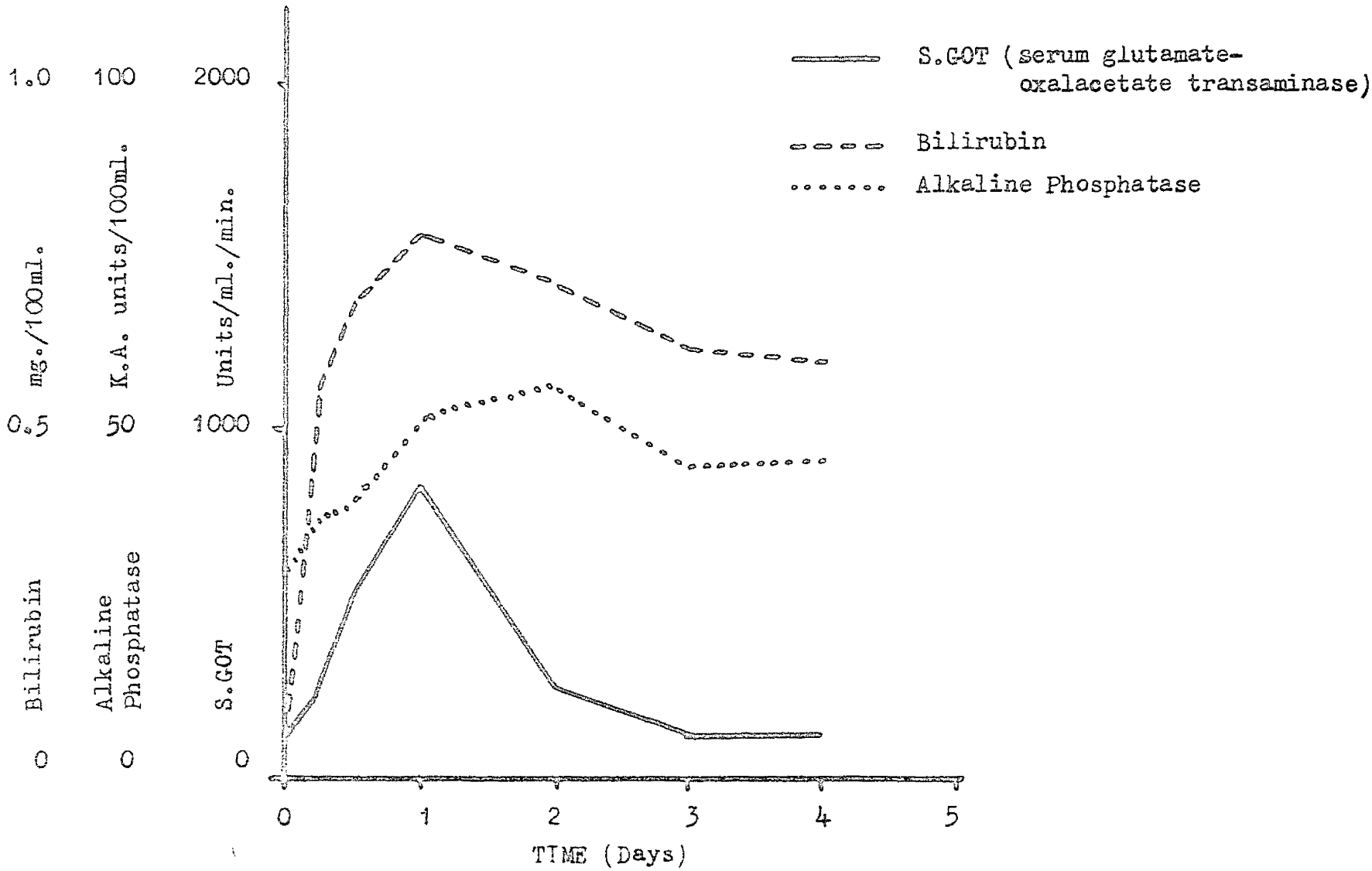
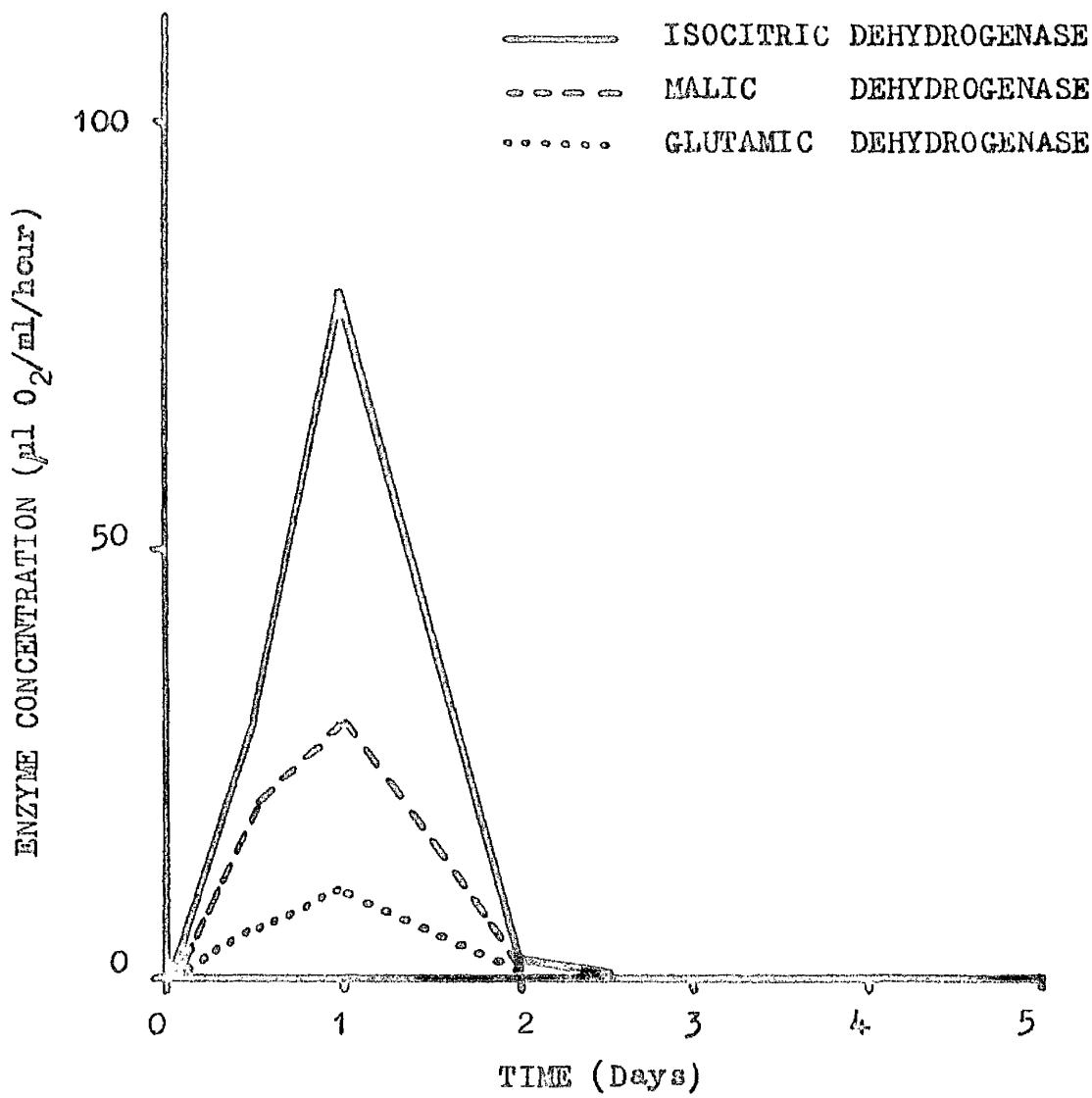

S.GOT (serum glutamate-oxalacetate transaminase)
Bilirubin
Alkaline Phosphatase
1.0
0.5
0
Bilirubin
mg./100ml.
100
50
0
Alkaline Phosphatase
K.A. units/100ml.
2000
1000
0
S.GOT
Units/ml./min.
0
1
2
3
4
5
TIME (Days)

FIGURE 12.

THE EFFECT OF THIOACETAMIDE POISONING

ON BLOOD ENZYMES:

(from data published by REES and SINHA, 1960).


ISOCITRIC DEHYDROGENASE
MALIC DEHYDROGENASE
GLUTAMIC DEHYDROGENASE
ENZYME CONCENTRATION (µl O_2/ml/hour)
100
50
0
0
1
2
3
4
5
TIME (Days)

MATERIAL AND METHODS.

The stock solution of Nembutal containing 60 mg. per ml. was diluted x10 with water immediately before use to provide doses of a convenient volume. The diluted solution was administered by intraperitoneal injection and the tests were carried out at the same time each day and under the same environmental conditions.

The times from injection to loss of the righting reflex and to recovery of the righting reflex was recorded. As the time required to induce narcosis was constant and short compared with the time it took for the animals to recover, it was found convenient to use the time from injection to recovery of the righting reflex as the standard measurement of the duration of action of the Nembutal. This period has been called the Narcosis Time and the procedure has been designated the Nembutal Narcosis Test.

RESULTS.

1. Liver Regeneration Following Partial Hepatectomy.

The standard partial hepatectomy was carried out on female Sprague-Dawley rats aged 49 days. The animals were divided into three groups by random selection and treated as follows:-

Group 1 - The animals were killed by a blow on the head and thereafter partial hepatectomy was performed. The weights of the freshly excised liver and the liver remaining in situ were measured.

Group 2 - Partial hepatectomy was carried out and the liver excised weighed. Twenty-four hours after operation the animals were killed and the weight of the remaining liver was recorded.

Group 3 - The weight of liver remaining three days after partial hepatectomy was recorded.

The results obtained in the experiment are shown in Tables 13, 14, 15, and 16, and Figure 13. It was found that partial hepatectomy removed 65% of the liver mass. By twenty-four hours after operation the liver remnant had regenerated to 40% of its original weight and by three days it had attained 72% of its original mass.

TABLE 13.

Liver Regeneration Following Partial Hepatectomy (Group 1)

Weight of liver excised on Day 0 G.	Weight of liver remaining on Day 0 G.	Total liver weight on Day 0 G.	% liver excised
4.55	2.6	7.15	63.6
3.5	1.8	5.3	66.0
3.9	1.5	5.4	72.2
3.1	2.0	5.1	60.8
3.6	2.1	5.7	63.2
4.45	2.65	7.1	62.6
3.8	2.1	5.9	64.4
4.75	1.6	6.35	74.8
2.9	2.3	5.2	55.8
3.2	1.7	4.9	65.3
3.77	2.04	5.81	65

TABLE 14.

Liver Regeneration Following Partial Hepatectomy (Group 2)

Weight of liver excised on Day 0 G.	Weight of liver remaining on Day +1 G.
4.0	2.9
5.0	2.7
5.3	3.1
4.0	2.8
5.0	3.2
4.9	2.9
6.8	3.9
4.5	2.4
4.3	2.9
3.9	2.4
4.77	2.92

TABLE 15.

Liver Regeneration Following Partial Hepatectomy (Group 3)

Weight of liver excised on Day 0 g.	Weight of liver remaining on Day +3 g.
4.3	6.0
5.1	4.7
5.0	5.1
5.9	4.8
4.3	4.3
3.9	4.0
3.5	5.7
4.5	5.9
4.3	5.7
4.5	3.9
4.53	5.01

TABLE 16.

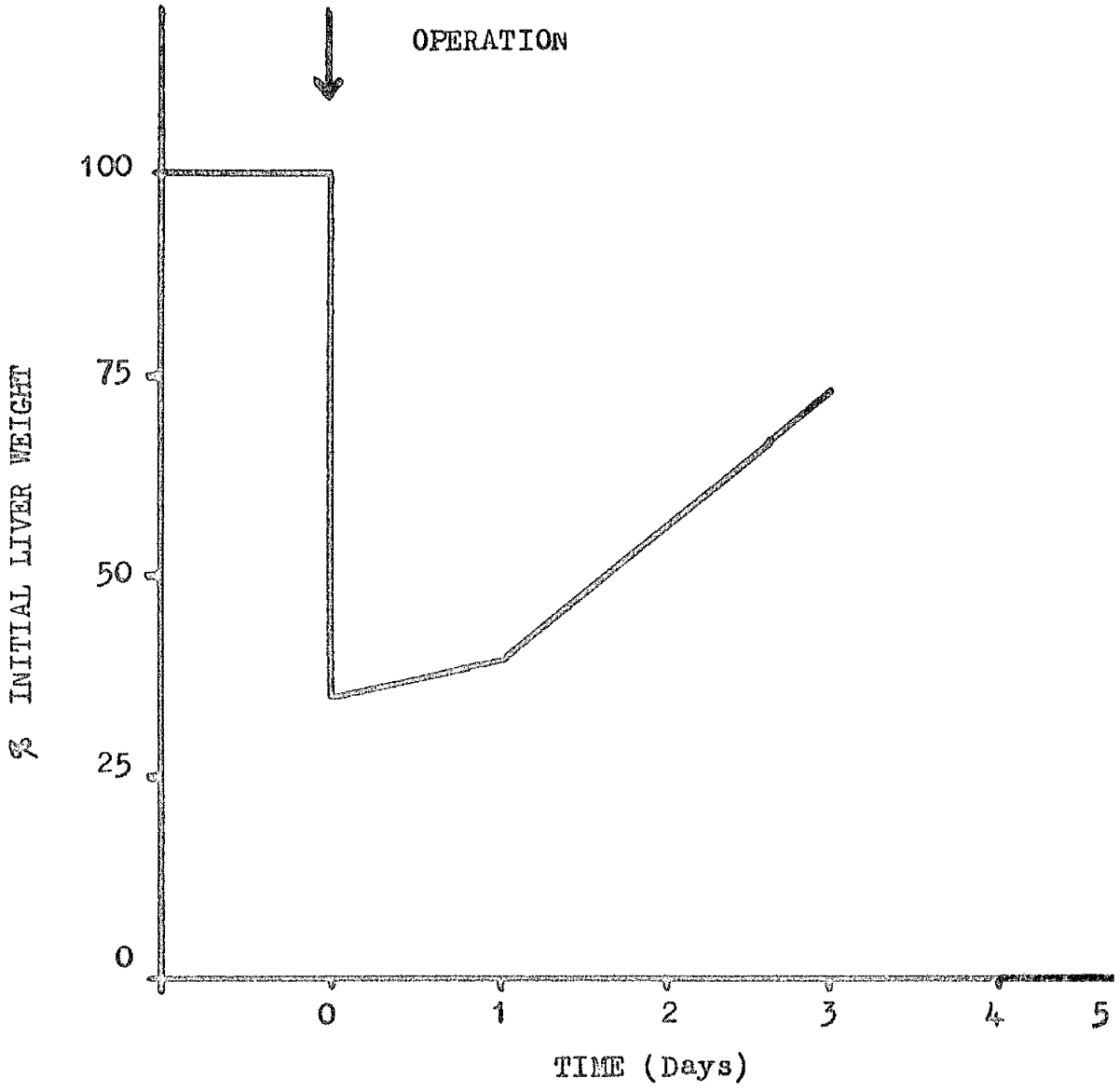
Liver Regeneration Following Partial Hepatectomy.

Interval Days after Partial Hepatectomy	Weight of Liver Excised g.	Weight of Liver Remaining g.	Total Liver Weight on Day 0 (Liver Weight Excised on Day 0 x 100/65)	Regeneration % of Total Liver Weight on Day 0 (Liver Weight Remaining on Day 1 or Day 3 x 100 Total Liver Weight on Day 0)
0	3.77	2.04	5.81	35
1	4.77	2.92	7.34	40
3	4.53	5.01	6.97	72

FIGURE 13.

LIVER REGENERATION FOLLOWING

PARTIAL HEPATECTOMY.


OPERATION
100
75
50
25
0
% INITIAL LIVER WEIGHT
0
1
2
3
4
5
TIME (Days)

2. The Effect of a Single Dose of Carbon Tetrachloride on the Duration of Nembutal Narcosis.

In the pilot study four male Sprague-Dawley rats aged 34 days each received a dose of 3 mg. Nembutal per 100 g. body weight by intraperitoneal injection and the duration of narcosis was measured. This procedure was carried out on three consecutive days to obtain basal values. Liver damage was then inflicted by a single intraperitoneal injection of 0.3 ml. of a solution of 50% carbon tetrachloride in olive oil. The narcosis times were then estimated daily for the next 5 days.

The results are recorded in Tables 17 and 18, and Figure 14. Although there was a marked increase in the duration of narcosis following treatment with carbon tetrachloride the time from injection of the Nembutal to loss of the righting reflex showed no significant variation and remained short when compared with the duration of narcosis.

The correlation between the narcosis times and other liver function tests (obtained from Rees and Sinha, 1960) following treatment with carbon tetrachloride is illustrated by Figure 15. The pattern of the changes are very similar.

TABLE 17.

The Effect of a Single Dose of Carbon Tetrachloride on the Duration of Nembutal Narcosis.

Time of Test Related to CCl_4 Days	Body Weight G.	Time from Injection to Induction of Narcosis (Minutes)	Time from Injection to Recovery from Narcosis (Minutes)
-3	104 106 95 108	3 4 3 4	62 59 72 65
-1	- - - -	3 4 4 6	54 46 62 63
0	112 119 108 118	4 5 4 5	40 37 57 38
1	102 112 100 -	5 5 5 6	177 240 232 Died
2	98 110 97 -	4 4 4 -	75 103 85 -
3	102 119 102 -	4 5 4 -	56 46 55 -
4	106 127 107 -	5 8 5 -	43 38 46 -
6	124 138 125 -	10 10 12 -	34 21 35 -

TABLE 18.

The Effect of a Single Dose of Carbon Tetrachloride on the Duration of Nembutal Narcosis.

Time of Test Related to CCl_4 Days	Average Body Weight Gm.	Average time from Injection to Induction of Narcosis (Minutes)	Average time from Injection to Recovery from Narcosis (Minutes)
-3	103	3.5	64.5
-1	-	4.25	56.25
0	114	4.5	43
1	105	5.2	216
2	102	4.0	87.7
3	108	4.3	52.3
4	113	6.0	42.3
6	129	10.7	30

FIGURE 14.

THE EFFECT OF A SINGLE DOSE OF CARBON TETRACHLORIDE ON THE DURATION OF NEMBUTAL NARCOSIS.

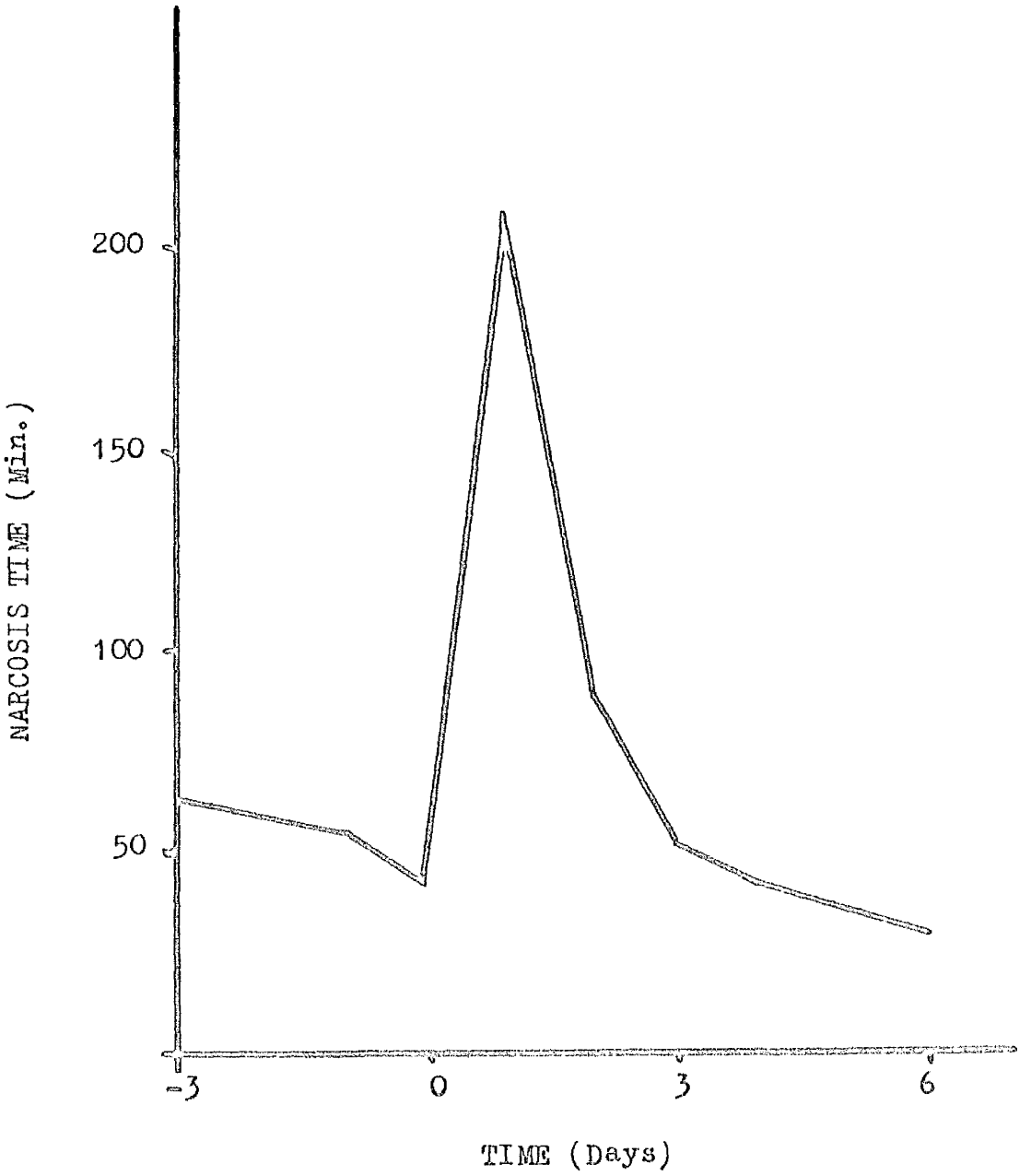
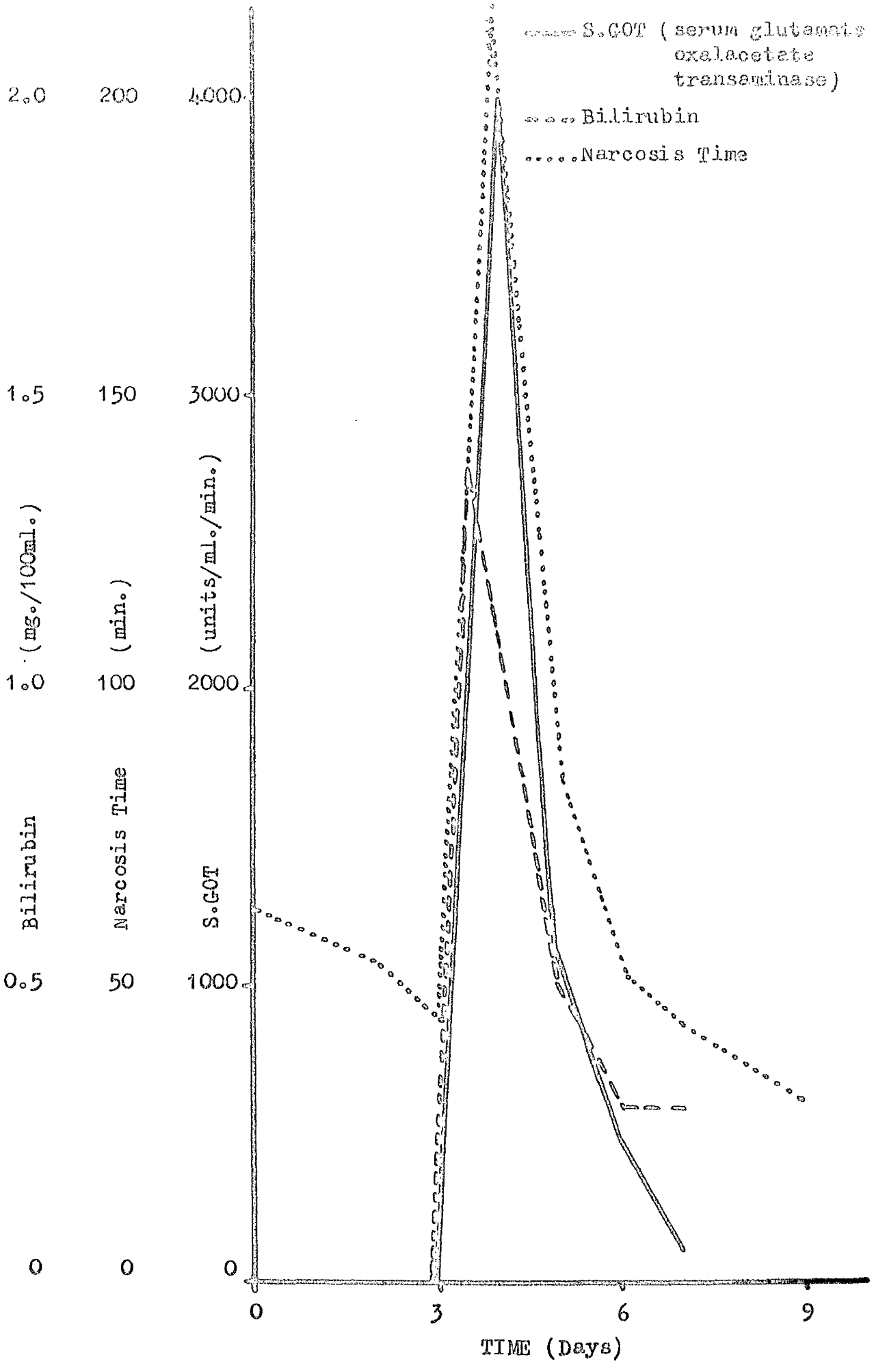

NARCOSIS TIME (Min.)
200
150
100
50
-3
0
3
6
TIME (Days)

FIGURE 15.

THE CORRELATION OF THE NARCOSIS TIMES AND LIVER FUNCTION TESTS (after REES and SINHA, 1960) FOLLOWING THE ADMINISTRATION OF CARBON TETRACHLORIDE.


S.GOT (serum glutamic oxalacetate transaminase)
Bilirubin
Narcosis Time
Bilirubin (mg./100ml.)
Narcosis Time (min.)
S.GOT (units/ml./min.)
2.0
1.5
1.0
0.5
0
200
150
100
50
0
4000
3000
2000
1000
0
0
3
6
9
TIME (Days)

3. The Effect of Various Doses of Nembutal on the Duration of Narcosis.

To determine the most convenient dosage of Nembutal for use in the measurement of narcosis time, various groups of rats received different dosage schedules. The average narcosis times were then plotted against the dosage of Nembutal used. The results are recorded in Tables 19 and 20. When plotted (Figure 16) it was found that the mid-point of the curve coincided with a dosage of 2.5 mg. Nembutal per 100 g. body weight, corresponding to a narcosis time of sixty minutes. This dosage was used for all subsequent experiments.

TABLE 19.

The Effect of Various Doses of Nembutal on the Duration of Narcosis.

Age of Rat (Days)	Body Weight g.	Nembutal mg.	Narcosis Time (Minutes)
34	104	3	62
34	106	3	59
34	95	3	72
34	108	3	65
39	104	3	80
39	120	3	62
39	122	3	97
43	133	3.99	149
46	138	1.5	0
46	149	1.5	0
46	122	1.5	0
46	156	1.5	0
46	150	1.5	0
46	164	5	100
46	139	4	Died
46	133	4	Died
47	143	2.0	0
47	150	2.0	0
47	127	2.0	0
47	164	2.0	0
47	153	2.0	0

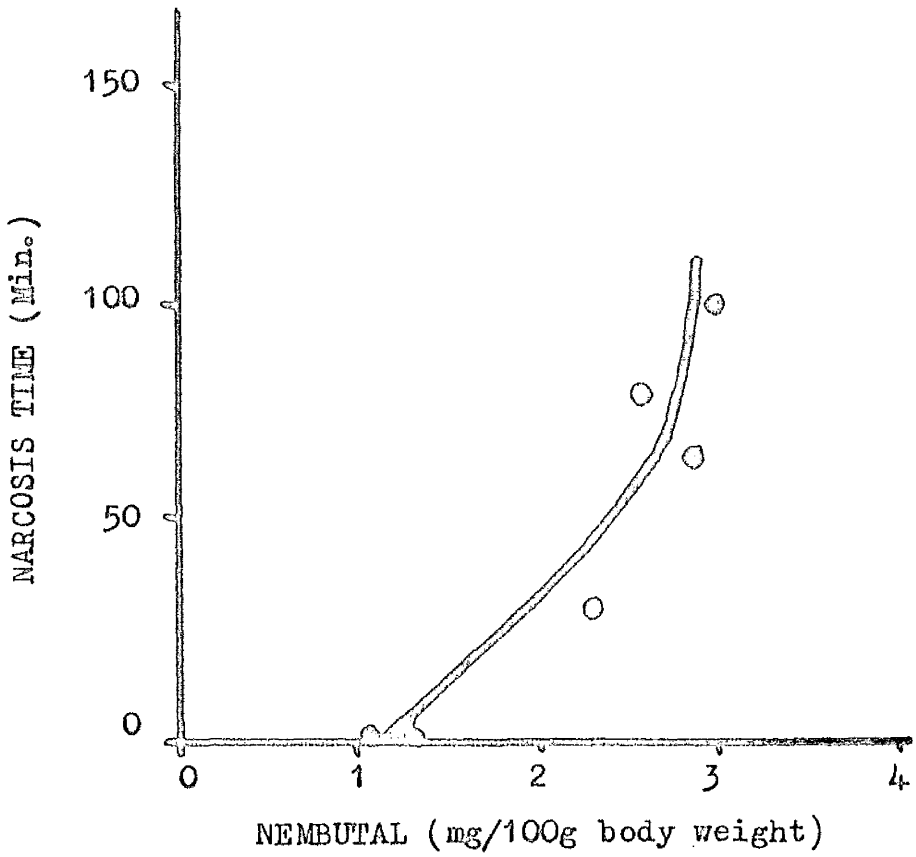
TABLE 20.

The Effect of Various Doses of Nembutal on the Duration of Narcosis.

Age of Rat (Days)	Average Body Weight g.	Nembutal mg./100 g. Body Weight.	Average Narcosis Time (Minutes)
46	143	1.05	0
47	147	1.36	0
43	129	2.33	30
39	115	2.61	79.7
34	103	2.91	64.5
43	133	3.0	149
46	145	3.0	100

FIGURE 16.

THE EFFECT OF VARIOUS DOSES OF NEMBUTAL ON THE DURATION OF NARCOSIS.


150
100
50
0
NARCOSIS TIME (Min.)
0
1
2
3
4
NEMBUTAL (mg/100g body weight)

4. The Effect of Various Doses of Carbon Tetrachloride on Nembutal Narcosis.

Having demonstrated that the narcosis time was related to other established forms of liver function test, the narcosis times were measured in rats subjected to various doses of carbon tetrachloride. It was found that larger doses of the hepatotoxic agent did produce longer narcosis times, as shown in Table 21 and Figure 17.

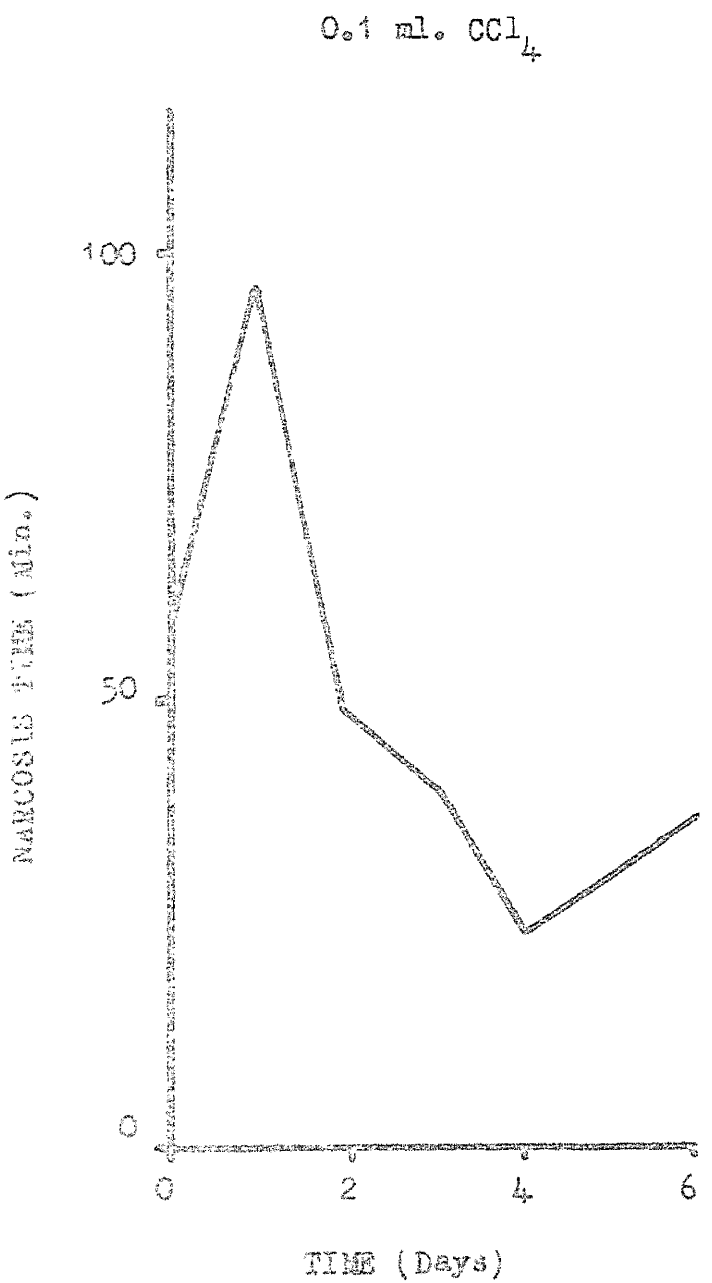
TABLE 21.

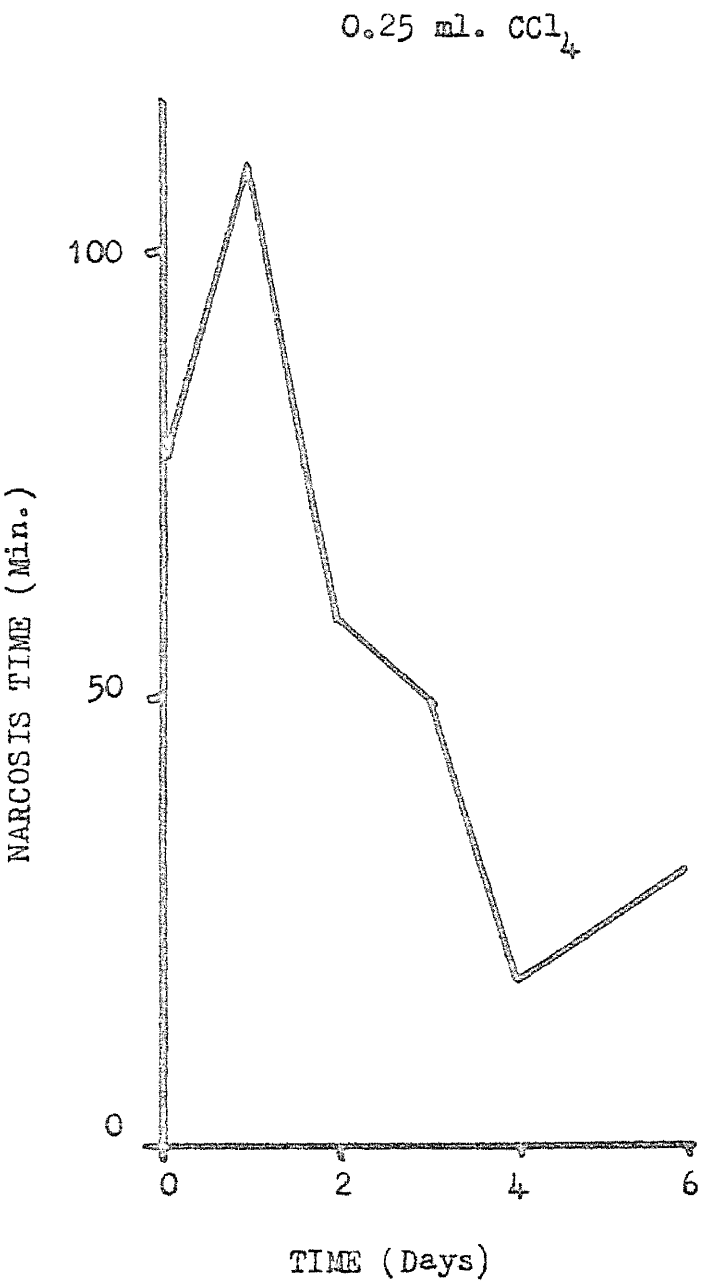
The Effect of Various Doses of Carbon Tetrachloride on Nembutal Narcosis.

Body Weight G.	Treatment ml. CCl_4 50% in Olive Oil	Daily Narcosis Times (Minutes)						
		0	1	2	3	4	5	6
124	0.1	80	97	52	53	0	-	44
165	0.1	32	95	41	30	36	-	36
157	0.1	75	100	55	54	35	-	22
149	0.1	62	97	49	40	24	-	37
160	0.25	70	100	63	48	39	-	46
161	0.25	73	120	55	53	0	-	36
160	0.25	71	110	59	50	19	-	41

FIGURE 17.

THE EFFECT OF VARIOUS DOSES OF CARBON TETRACHLORIDE ON THE DURATION OF NEMBUTAL NARCOSIS.


0.1 ml. CCl_4
NARCOSIS TIME (Min.)
100
50
0
0
2
4
6
TIME (Days)


0.25 ml. CCl_4
NARCOSIS TIME (Min.)
100
50
0
0
2
4
6
TIME (Days)

5. The Effect of Repeated Doses of Nembutal on the Duration of Narcosis in the Intact Rat.

Since the purpose of the Nembutal Narcosis Test was to follow changes in liver function over a period of days it was of importance to investigate the effect of repeated daily doses of Nembutal on the narcosis time in intact rats.

This study was carried out on 51 day old female Sprague-Dawley rats receiving 2.5 mg. Nembutal per 100 g. body weight.

It was observed that the duration of narcosis time decreased each day until a basal level was reached on the second day, as shown in Table 22 and Figure 18.

TABLE 22.

The Effect of Repeated Doses of Nembutal on the Duration of Narcosis.

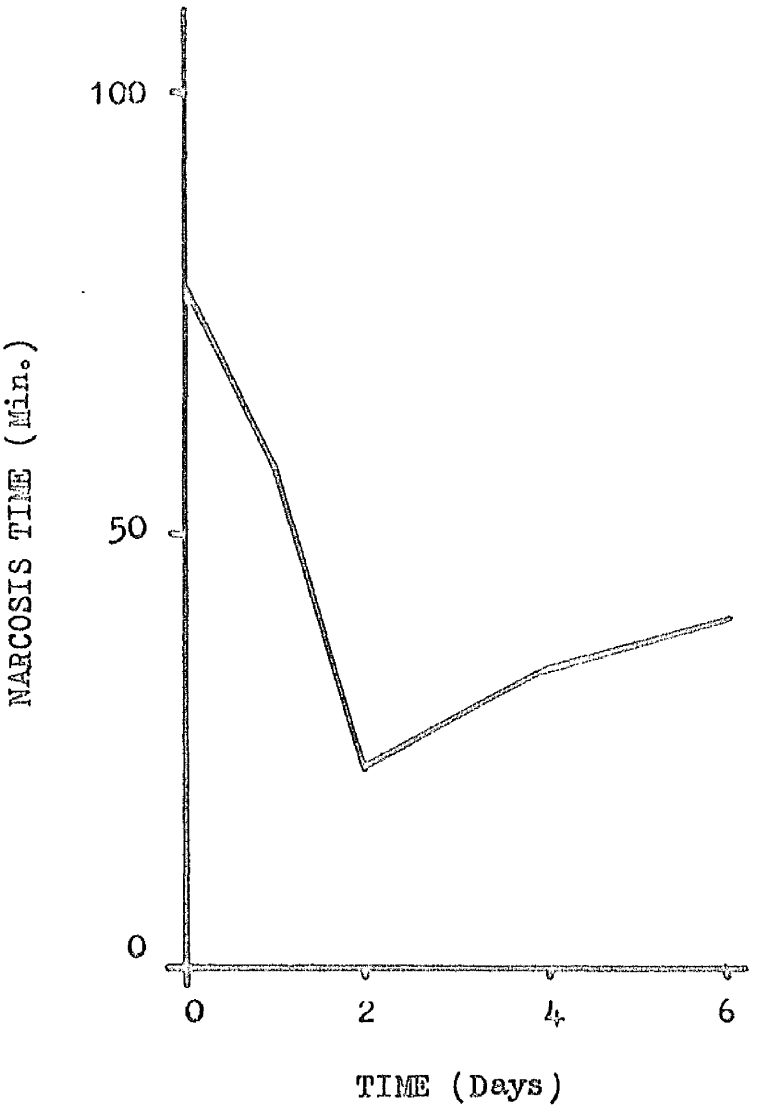
Body Weight G.	Daily Narcosis Time (Minutes)						
	0	1	2	3	4	5	6
155	80	60	40	33	27	-	60
168	70	60	0	25	33	-	28
165	80	55	30	30	43	-	32
163	77	58	23	29	34	-	40

FIGURE 18.

THE EFFECT OF REPEATED DOSES OF NUMBUTAL

(2.5 mg/100 G. body weight)

ON THE DURATION OF NARCOSIS.


100
50
0
NARCOSIS TIME (Min.)
0
2
4
6
TIME (Days)

6. The Effect of Partial Hepatectomy on the Duration of Nembutal Narcosis.

The standard form of partial hepatectomy was carried out on a group of female Sprague-Dawley rats aged 50 days. A similar group of rats was not subjected to operation and served as a control series for this experiment and for the experiments to determine the effect of dl-ethionine and glycine on hepatic function.

The narcosis time was measured under uniform conditions but for this particular group of experiments a standard dose of 4 mg. Nembutal was administered; this was equivalent to an average dose of 3 mg. Nembutal per 100 g. body weight.

The results are recorded in Table 23 and Figures 19 and 20.

It was found that partial hepatectomy produced a marked impairment of liver function. The maximum effect was apparent 24 hours after operation and control levels were reached 3 days after operation.

TABLE 23.

The Effect of Partial Hepatectomy on the Duration of Nembutal Narcosis.

Body Weight g.	Treatment on Day 0	Daily Narcosis Times (Minutes)							
		-3	-2	-1	0	1	2	3	4
130	Control	100	58	-	0	28	45	25	45
134		119	49	-	48	22	30	28	25
123		112	56	-	58	37	45	42	50
158		87	53	-	30	18	37	30	18
125		120	59	-	60	21	33	15	25
143	Partial Hepatectomy	104	40	-	-	98	67	41	40
156		81	53	-	-	124	72	37	47
133		88	48	-	-	118	65	39	27
162		82	48	-	-	106	64	30	28
137		114	52	-	-	126	73		0

FIGURE 19.

THE EFFECT OF REPEATED DOSES OF NEMBUTAL

(4.0 mg/100 G. body weight)

ON THE DURATION OF NARCOSIS.

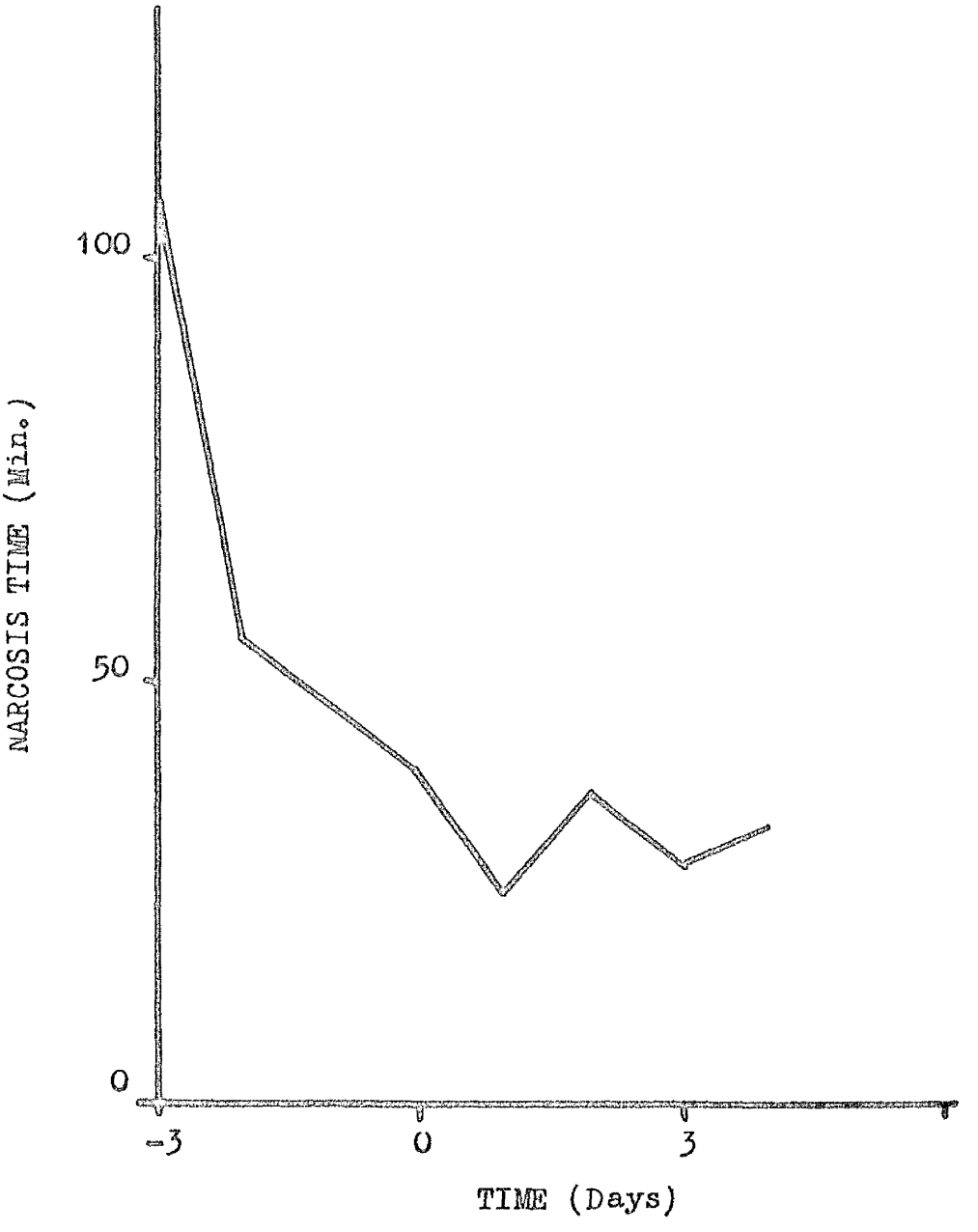
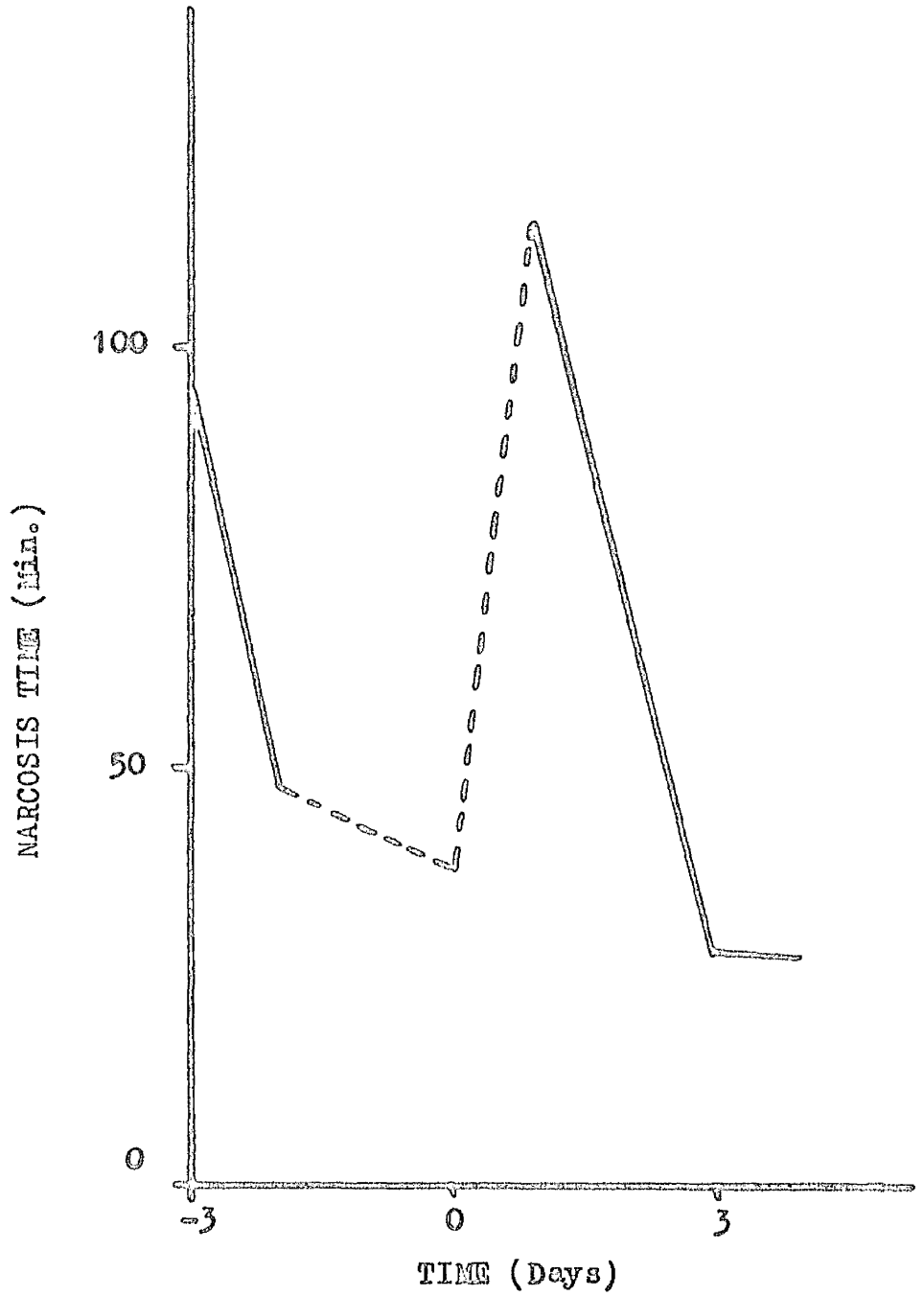

NARCOSIS TIME (Min.)
100
50
0
-3
0
3
TIME (Days)

FIGURE 20.

THE EFFECT OF PARTIAL HEPATECTOMY ON THE DURATION OF NEMBUTAL NARCOSIS.


100
50
0
NARCOSIS TIME (Min.)
-3
0
3
TIME (Days)

7. The Effect of dl-Ethionine on the Duration of Nembutal Narcosis.

The dl-ethionine was dissolved in normal saline to give a concentration of 25 mg. per 1 ml. of solution. The animals to be tested were starved overnight and received the amino acid by intraperitoneal injection the following morning. A total dose of 150 mg. (i.e. 1 mgm. per 1 g. body weight approximately) was given, two equal divided doses at 9 a.m. and 11 a.m., normal feeding being resumed at 2 p.m.

The narcosis times were estimated daily except on the day immediately preceding and on the actual day of administration of the amino acid.

It was found that dl-ethionine caused some increase in the narcosis time but the effect was not as pronounced as with partial hepatectomy. The maximum duration of narcosis occurred 48 hours after treatment with dl-ethionine compared with peak values 24 hours after partial hepatectomy. Recovery of liver function was also more prolonged (Table 24 and Figure 21).

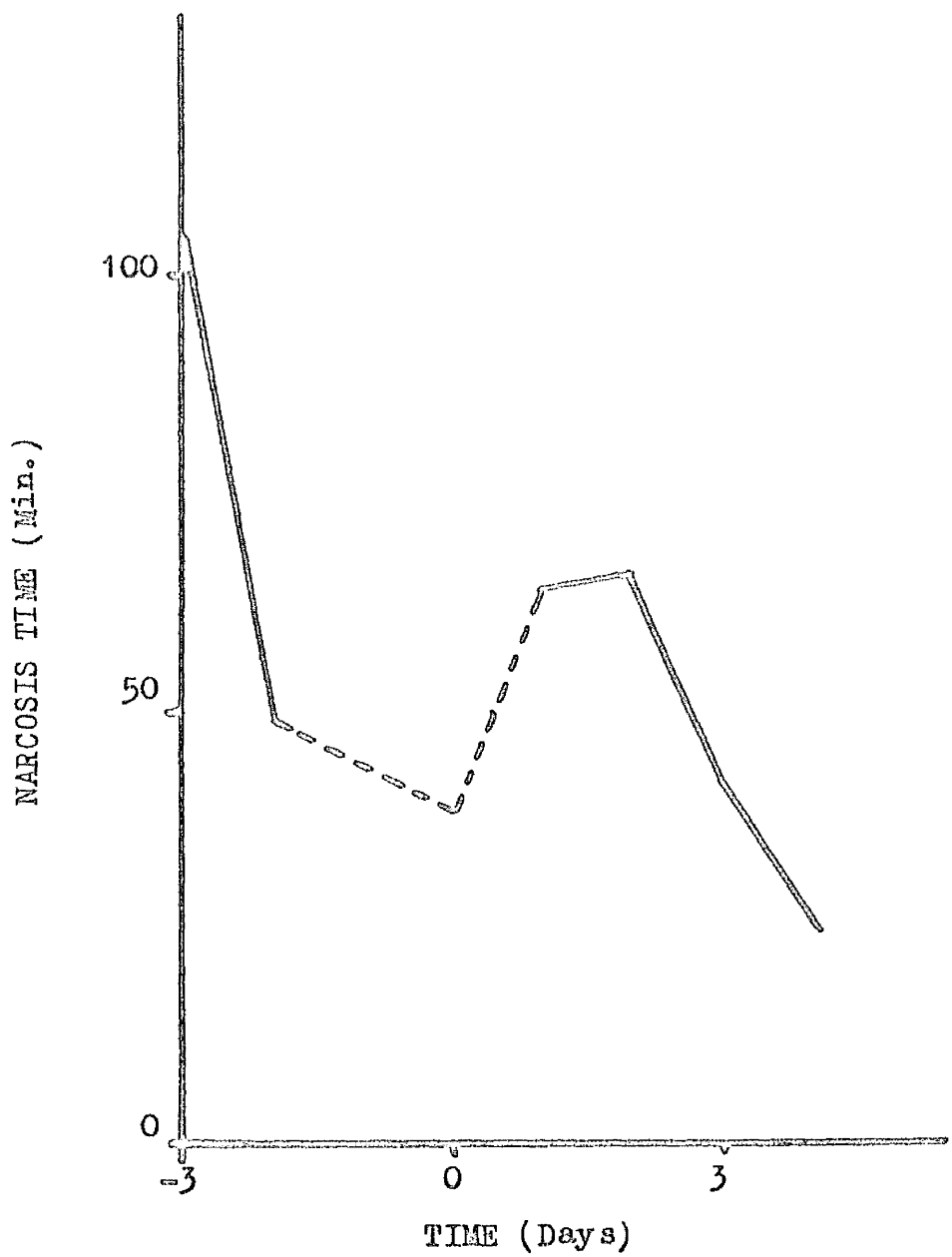
TABLE 24.

The Effect of dl-Ethionine on the Duration of Nembutal Narcosis.

Body Weight G.	Treatment on Day 0	Daily Narcosis Times (Minutes)							
		-3	-2	-1	0	1	2	3	4
133	dl-Ethionine	93	42	-	-	64	50	23	12
138		102	45	-	-	48	50	25	16
180		88	44	-	-	16	54	48	22
117		126	67	-	-	113	90	63	45
123		106	56	-	-	78	80	44	29

FIGURE 21.

THE EFFECT OF dl-ETHIONINE ON THE DURATION OF NEMBUTAL NARCOSIS.


NARCOSIS TIME (Min.)
100
50
0
-3
0
3
TIME (Days)

8. The Effect of Glycine on the Duration of Nembutal Narcosis.

A similar group of rats received glycine administered in the same dosage schedule and under the same conditions as dl-ethionine (150 mg. intraperitoneally).

The results of the effect of such treatment on the narcosis times is recorded in Table 25 and Figure 22. It is apparent that glycine has caused impairment in liver function. The degree of damage is similar to that produced by dl-ethionine. Recovery of liver function, however, is more rapid.

To compare the effects of partial hepatectomy, dl-ethionine and glycine, the narcosis times for each form of treatment have been expressed as a percentage of the control values obtained during the experiment on partial hepatectomy (Tables 26 and 27) and the results plotted in Figure 23.

TABLE 25.

The Effect of Glycine on the Duration of Nembutal Narcosis.

Body Weight G.	Treatment on day 0	Daily Narcosis Times (Minutes)							
		-3	-2	-1	0	1	2	3	4
111	Glycine	127	58	-	-	98	46	41	0
140		94	40	-	-	38	26	24	30
145		109	44	-	-	20	32	15	25
144		102	45	-	-	38	35	28	24
125		109	50	-	-	93	40	33	35

FIGURE 22.

THE EFFECT OF GLYCINE ON THE DURATION OF NEMBUTAL NARCOSIS.

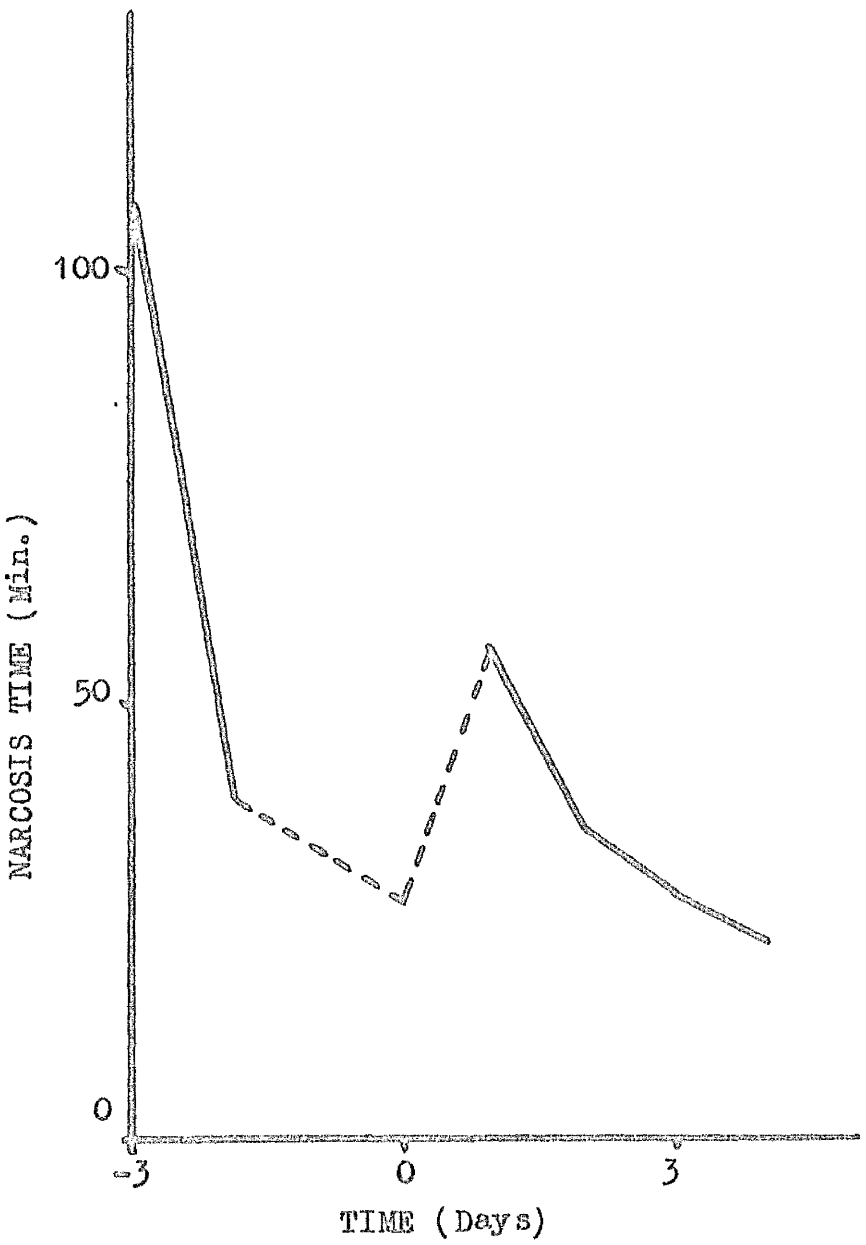

100
50
0
NARCOSIS TIME (Min.)
-3
0
3
TIME (Days)

TABLE 26.

The Effect of Partial Hepatectomy, dl-Ethionine and Glycine on Nembutal Narcosis.

Average Body Weight G.	Treatment on Day 0	Average Daily Narcosis Times (Minutes)							
		-3	-2	-1	0	1	2	3	4
134	Control	108	55	-	39	25	38	28	35
146	Partial Hepatectomy	94	48	-	-	114	68	29	28
138	dl-Ethionine	103	51	-	-	64	65	41	25
133	Glycine	108	47	-	-	57	36	28	23

TABLE 27.

The Effect of Partial Hepatectomy, dl-Ethionine and Glycine on Nembutal Narcosis.

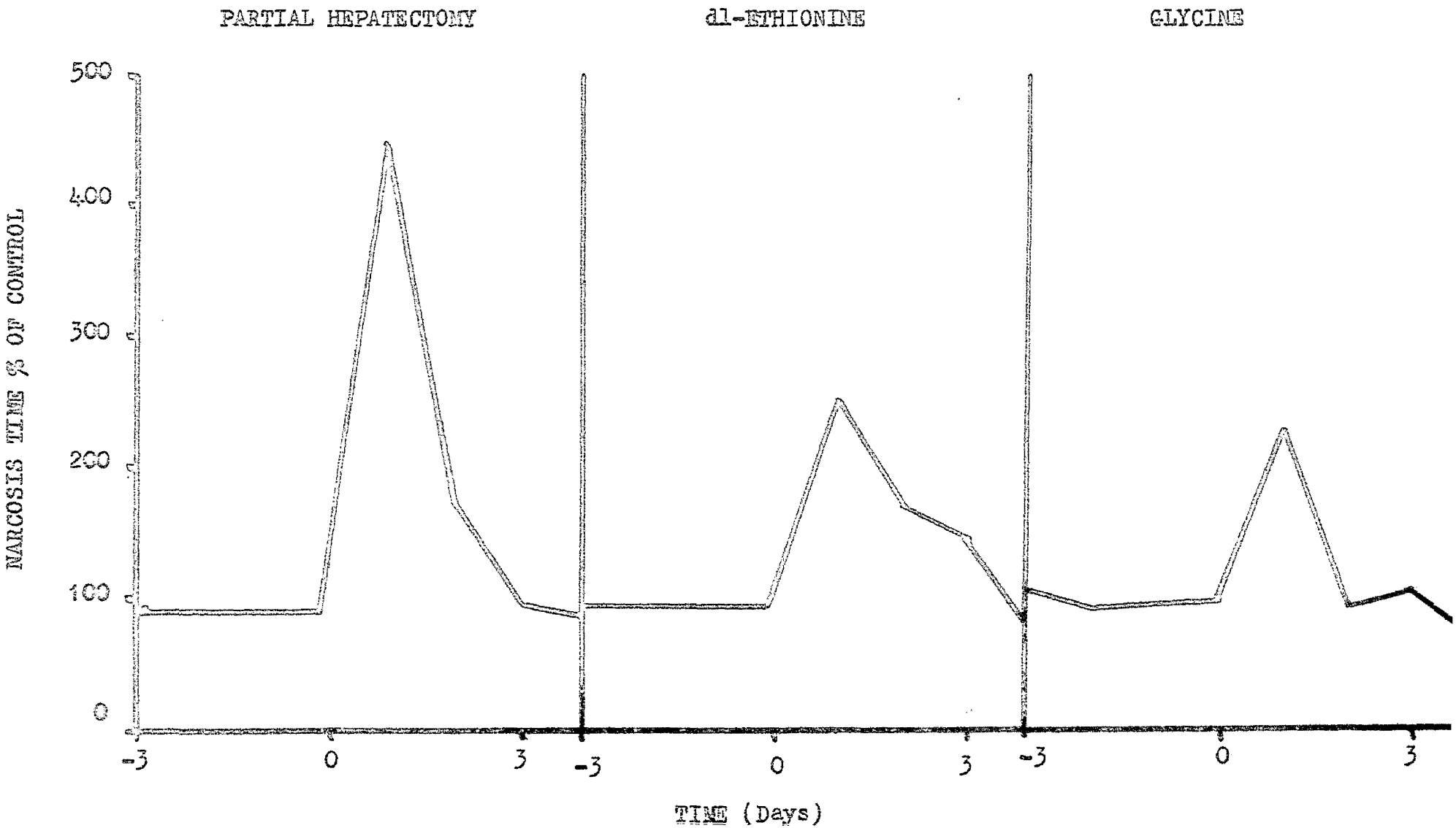
Treatment	Daily Narcosis Time % of Control Values							
	-3	-2	-1	0	1	2	3	4
Control	100	100	-	100	100	100	100	100
Partial Hepatectomy	87	88	-	-	454	179	105	87
dl-Ethionine	96	93	-	-	253	171	145	76
Glycine	101	86	-	-	228	94	101	70

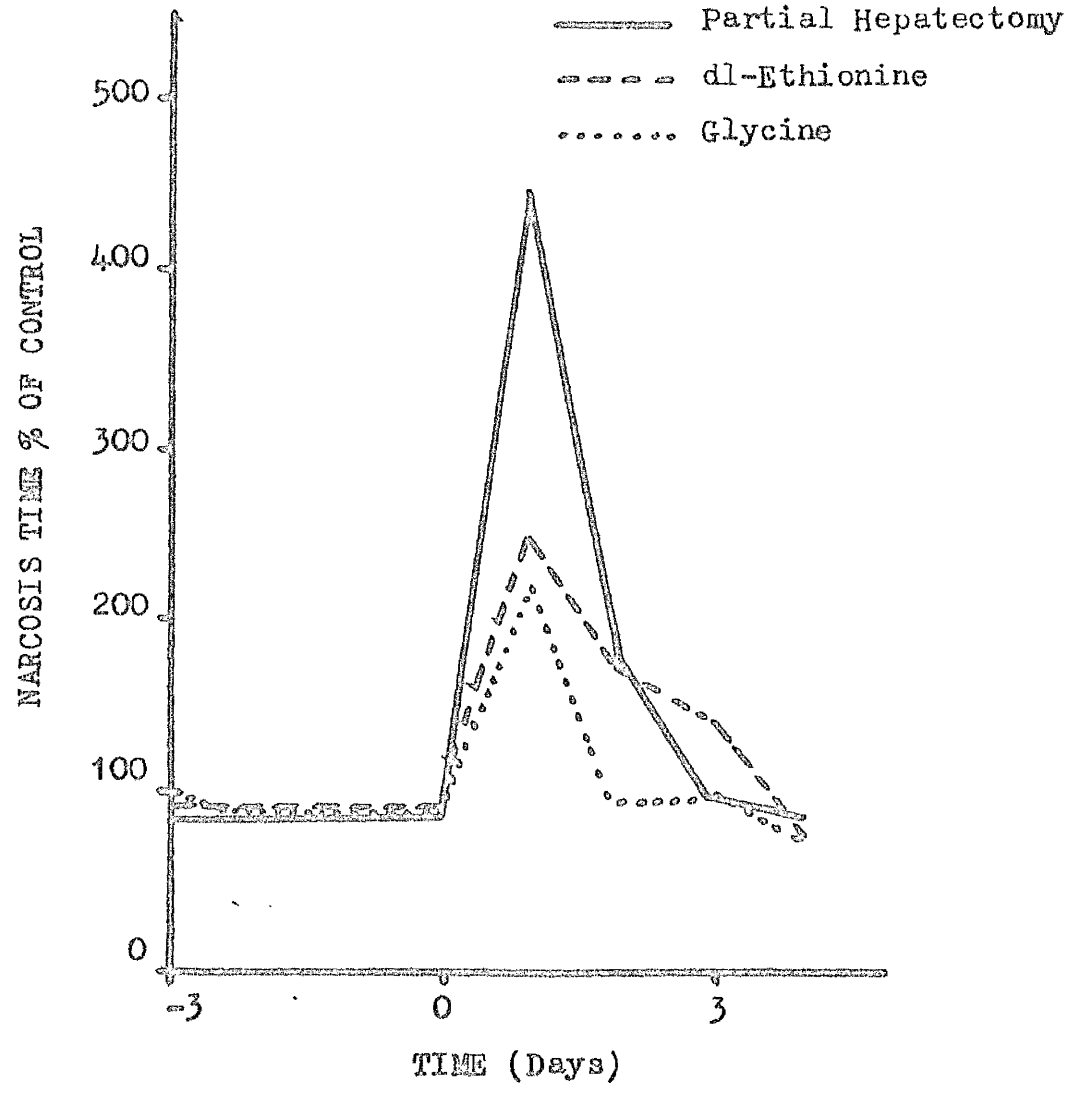
FIGURE 23.

THE EFFECT OF PARTIAL HEPATECTOMY,

dl-ETHIONINE, AND GLYCINE

ON THE DURATION OF NEMBUTAL NARCOSIS.


PARTIAL HEPATECTOMY
dl-ETHIONINE
GLYCINE
NARCOSIS TIME % OF CONTROL
500
400
300
200
100
0
-3
0
3
-3
0
3
-3
0
3
TIME (Days)


Partial Hepatectomy
dl-Ethionine
Glycine
NARCOSIS TIME % OF CONTROL
500
400
300
200
100
0
-3
0
3
TIME (Days)

9. The Effect of Thioacetamide on the Duration of Nembutal Narcosis.

Female Sprague-Dawley rats aged 50 days received thioacetamide 200 mg./Kg. body weight by intraperitoneal injection. The narcosis time was estimated using a test dose of Nembutal 2.5 mg./100 g. body weight at various times, ranging from 0 to 24 hours following the administration of the thioacetamide. The results are shown in Tables 28 and 29, and Figure 24.

It is apparent that thioacetamide does produce liver damage but the effect is short lived, being maximal at 6 hours after treatment. Liver function has recovered from the effects of thioacetamide by 24 hours.

TABLE 28.

The Effect of Thioacetamide on the Duration of Nembutal Narcosis.

Body Weight g.	Time after Thioacetamide (Hours)	Narcosis Time (Minutes)
183 140 156	0	49 80 82
125 144 157	2	104 88 123
140 119 168	6	118 140 119
136 162 174	18	91 92 99
150 150 170 160 140	24	95 41 84 94 80

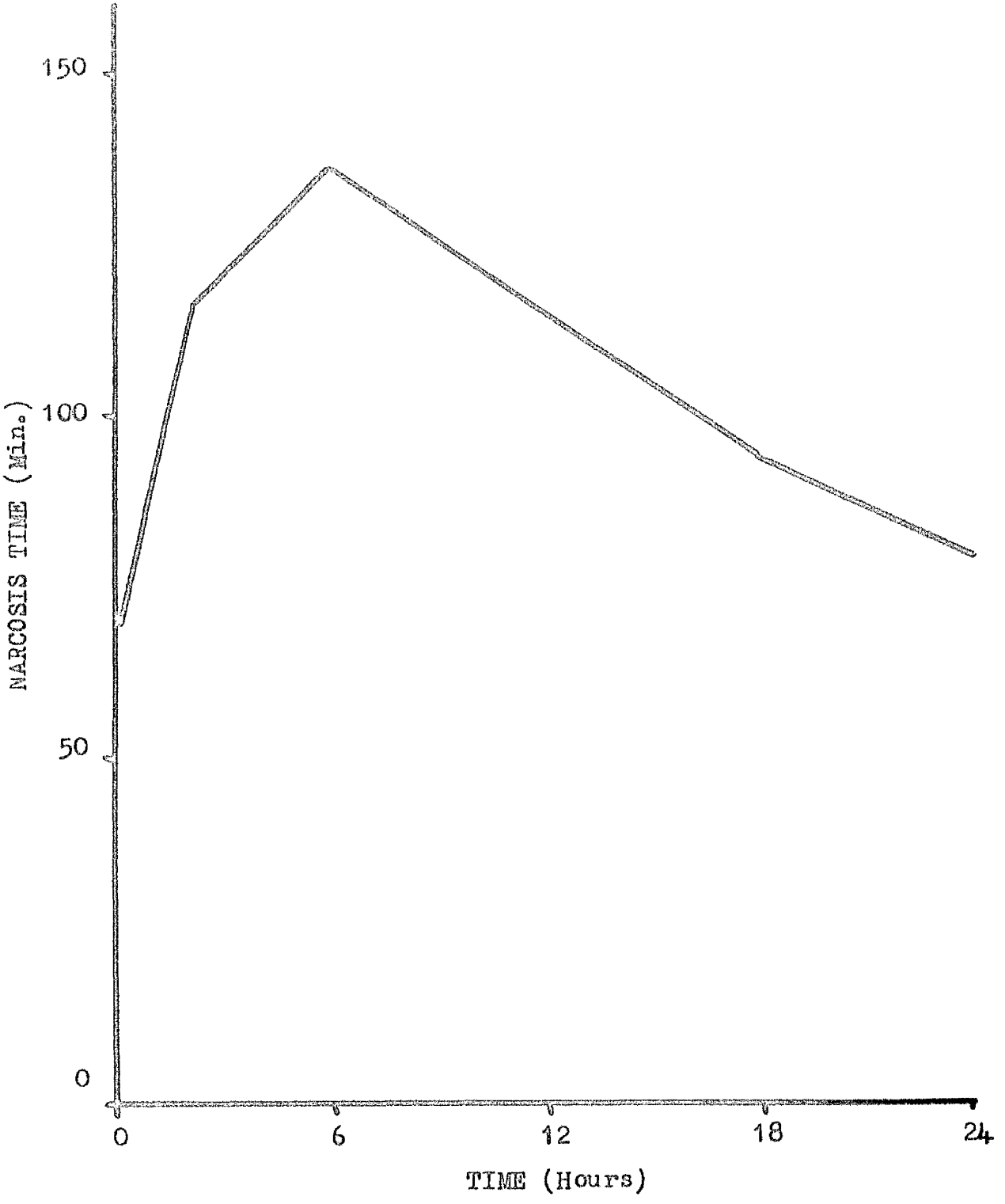
TABLE 29.

The Effect of Thioacetamide on the Duration of Nembutal Narcosis.

Average Body Weight g.	Time After Thioacetamide (Hours)	Average Narcosis Time (Minutes)
160	0	70
142	2	105
142	6	126
157	18	94
154	24	79

FIGURE 24.

THE EFFECT OF THIOACETAMIDE ON THE DURATION OF NEMBUTAL NARCOSIS.


150
100
50
0
NARCOSIS TIME (Min.)
0
6
12
18
24
TIME (Hours)

10. The Effect of Age and Weight on the Duration of Nembutal Narcosis.

The narcosis times were estimated in female rats of various ages using a standard dose of 2.5 mg./100 g. body weight of Nembutal. The weights of the rats were also recorded as shown in Tables 30 and 31.

When the duration of narcosis is plotted against the age of the rat (Figure 25) it is revealed that the immature animal is more susceptible to the narcotic effect of Nembutal than in the adult. The shortest narcosis times are found in animals aged 30 to 40 days.

Although the dosage of Nembutal used was proportional to the weight of the rat it was considered that the effect of Nembutal might be a function of the body weight rather than of the age of the rat. The growth curves (Figure 26) of these animals, however, do not show any variation which can be correlated with the changes observed in narcosis times in animals aged less than 50 days. Age, rather than weight, appears to be the critical factor.

TABLE 30.

The Effect of Age and Weight on the Duration of Nembutal Narcosis (1)

Age (Days)	Body Weight g.	Narcosis Time (Minutes)
3	6	146
3	6	147
3	6	164
10	20	110
10	17	173
10	15	174
10	15	164
10	20	171
15	36	126
15	35	141
15	34	140
15	35	131
15	33	142
16	24	151
16	23	157
16	24	154
20	35	80
20	34	74
20	31	76
20	28	77
20	35	80
23	38	95
23	40	94
23	41	81
25	70	72
25	68	70
25	65	71
25	70	70
25	69	70
31	74	69
31	80	65
31	70	69

TABLE 30 (contd.)

Age (Days)	Body Weight g.	Narcosis Time (Minutes)
31	79	70
31	60	76
39	44	79
39	40	38
49	137	92
49	148	91
49	143	104
52	172	91
52	194	70
52	157	89
102	250	101
102	240	88
102	245	99
105	225	98
105	240	79
105	235	89
141	250	93
141	235	111
152	270	74
152	270	89
173	330	87
173	300	99
173	325	100
207	270	83
207	280	100
207	275	99
214	340	84
214	320	93
214	330	100
220	282	80
240	279	80
240	262	87

TABLE 31.

The Effect of Age and Weight on the Duration of Nembutal Narcosis (2).

Age (Days)	Average Body Weight g.	Average Narcosis Time (Minutes)
3	6	152
10	17.4	158
15	34.6	136
16	23.7	154
20	32.6	77
23	39.7	90
25	68.4	71
31	72.6	70
39	42	58
49	142.7	96
52	174.3	83
102	245	96
105	233.3	89
141	242.5	102
152	270	82
173	318.3	96
207	275	94
214	330	92
220	282	80
240	270.5	84

FIGURE 25.

THE EFFECT OF AGE ON THE DURATION OF NEMBUTAL NARCOSIS.

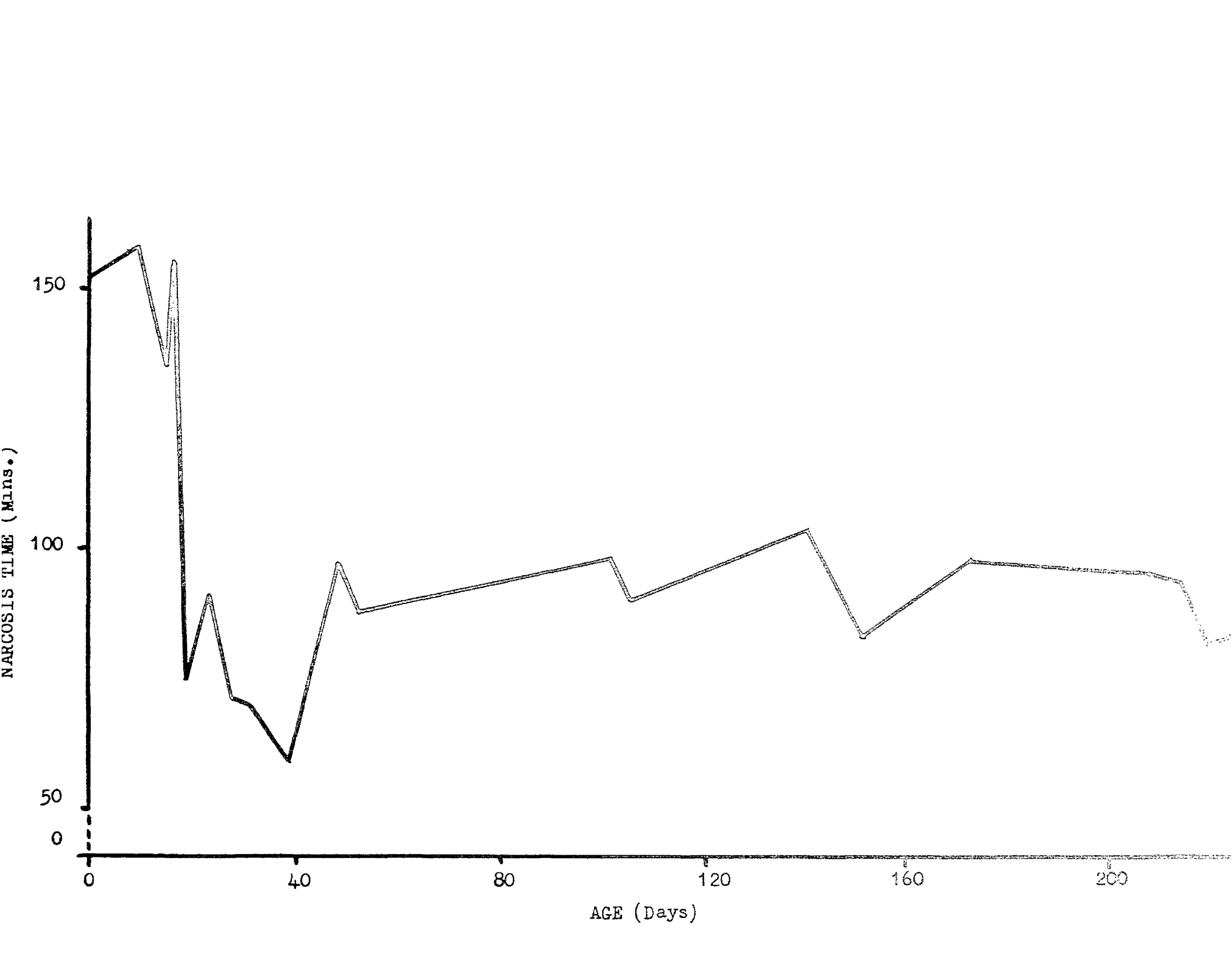
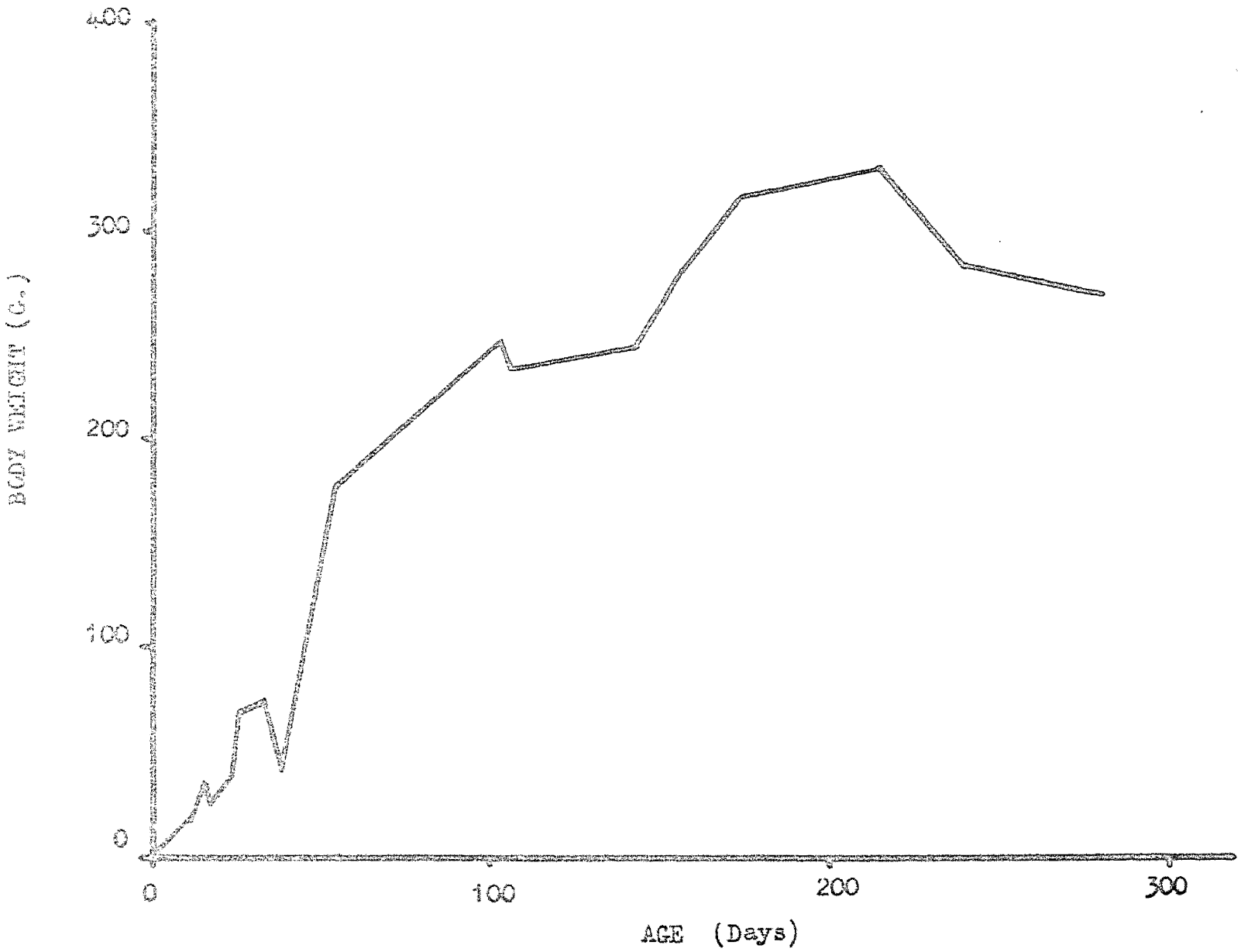

NARCOSIS TIME (Mins.)
150
100
50
0
0
40
80
120
160
200
AGE (Days)

FIGURE 26.

THE RELATIONSHIP OF BODY WEIGHT TO AGE

IN THE FEMALE SPRAGUE-DAWLEY RAT.


400
300
200
100
0
BODY WEIGHT (G.)
0
100
200
300
AGE (Days)

DISCUSSION.

A standard form of partial hepatectomy has been found to remove 65% of the liver mass, which agrees with the figures previously reported (Huggins and Anderson, 1931). When the rate of liver regeneration (Figure 13) is compared with the variation in narcosis times following partial hepatectomy (Figure 23), an inverse relationship is apparent. Moreover, the narcosis times following carbon tetrachloride treatment have also been found to correspond closely with the changes that occur in other liver function tests, (Figure 15). These results indicate that the duration of Nembutal narcosis is a valid quantitative liver function test. It is a convenient method, being easy to use and suitable for following the course of liver damage over a period of time in individual animals. Although it is a biological measurement and shows variations between individual animals, the average values have been found to be reproducible.

This test was applied to the study of those factors which influenced the adrenolytic action of DMBA. As expected dl-ethionine, which is hepatotoxic, produced an increase in the duration of narcosis. The fact that it was a poor protector against DMBA could be correlated with the evidence that it caused less liver damage than did either partial hepatectomy or carbon tetrachloride (Figure 23).

Glycine had been found to give, unexpectedly, protection against DMBA. The mechanism of this action was uncertain. However, it has now been shown that glycine produces liver damage

as revealed by the Nembutal Narcosis Test (Figure 22). This impairment could well account for its protective capacity against DMBA. Its action is therefore comparable to that of other forms of liver damage. It is suggested that the liver damage is brought about by ammonia intoxication resulting from the metabolism of an excessive quantity of glycine.

Investigation of thioacetamide has revealed that it is a weak liver poison in the dosage used for these experiments and that its effect is short lived (Figure 24). This corresponds to the fact that thioacetamide provides only mild protection against DMBA and that this protection is limited to a critical period of short duration around 6 hours following the DMBA treatment (Figure 3).

These time relationships suggest that the critical time for interference with the metabolism of DMBA if its adrenolytic action is to be prevented, is about 12 hours after is intravenous injection. However, when carbon tetrachloride is administered after DMBA adrenal protection was not obtained (Figure 2). Not only is carbon tetrachloride potent liver poison, but it is also rapid in action with evidence of functionl damage to the liver mitochondria within 3 hours (Smuckler, Iseri and Benditt, 1964). Thioacetamide is reported as both producing histological damage to the liver (Gupta, 1956) and changes in the serum enzymes (Rees, Sinha and Spector, 1961) at 6 hours. A delayed onset of action of carbon tetrachloride cannot be invoked to account for the lack of protection when it is given

after DMBA and so far no explanation can be offered to account for this difference in the action of carbon tetrachloride and thioacetamide.

The importance of the age of the rat relative to its susceptibility to adrenal necrosis has been noted and it has been suggested that the ability of the adrenal to synthesize corticosterone is critical.

The Nembutal Narcosis Test however, has raised the possibility that variation in liver function with age might be involved. Immature animals are known to have liver enzyme systems which are less efficient than adults (Dawkins, 1966) and this could explain the variation in narcosis times. It is possible that a similar process could interfere with DMBA metabolism and thus prevent adrenal damage. Doubt has also been cast upon the importance of corticosterone synthesis by other workers. Thus Metopirone has been shown to increase the rate of metabolism of DMBA by hydroxylation of its methyl groups (Boyland and Sims, 1967) and could, therefore, bring about a more rapid detoxification of the adrenolytic derivative. Metopirone has also been shown to bring about adrenal protection under conditions in which there is no apparent change in corticosterone synthesis (Jellinick, Garland and McRitchie, 1968). It is of interest that Metopirone will also enhance the detoxification of Nembutal (Wheatley, 1968ii). Inspection of Figure 28 reveals, however, that the narcosis times at 20 days

of age are similar to the adult values. If liver function was the only factor involved then these animals should be susceptible to the adrenolytic effect of DMBA - but this has never been shown to be the case.

Investigation of the metabolism of DMBA by immature rats has shown that DMBA, 7-OHM-12-MBA, and 12-OHM-7-MBA are all metabolised more quickly by rats aged between 15 and 25 days (Sims and Grover, 1967). The peak rates of metabolism of both DMBA and Nembutal are found to coincide at about 35 days. It has been suggested that the temporary increase in the metabolism of DMBA is related to the induction of microsomal enzymes by dietary components during weaning (Sims and Grover, 1967).

The Nembutal Narcosis Test has utilised the metabolism of Nembutal to investigate procedures which influence the metabolism of DMBA. There is an apparent association between the ability of a rat to metabolise both DMBA and Nembutal at particular periods in its life. The evidence suggests, therefore, that a common enzyme system may be involved in both cases. Such a view is strengthened by finding that treatment with Nembutal will act as a protector against DMBA-induced adrenal necrosis (Figure 6), the most likely explanation being that the enzymes induced by Nembutal are capable of bringing about a rapid elimination of the DMBA before it has time to damage the adrenal.

GENERAL SUMMARY.

The polycyclic hydrocarbon DMBA possesses a unique adrenolytic property and it is also a potent carcinogen.

Evidence has been presented that the adrenolytic effect can be prevented by interference with liver function suggesting that a metabolite of DMBA is the active adrenolytic agent and not the parent compound.

Enhancement of liver enzyme systems, e.g. by Nembutal, will also provide protection and it is postulated that the active metabolite can itself be inactivated by such enzymes.

Treatments that have been shown to provide efficient protection for the adrenals have not prevented carcinogenesis. Hence, the adrenolytic and carcinogenic effects can be dissociated. It is postulated that the carcinogenic effect is due to DMBA itself.

Investigation of liver function has shown that the degree of hepatic damage can be correlated with the degree of adrenal protection that various treatments bring about. Although the immature rat has been found to have deficient liver function as regards drug detoxification when compared with the adult animal, the evidence provided by the Nembutal Narcosis Test suggests that this fact does not adequately explain the resistance of the immature rat to DMBA-induced adrenal necrosis. Active synthesis of corticosterone by the adrenal gland is still considered to be of critical importance.

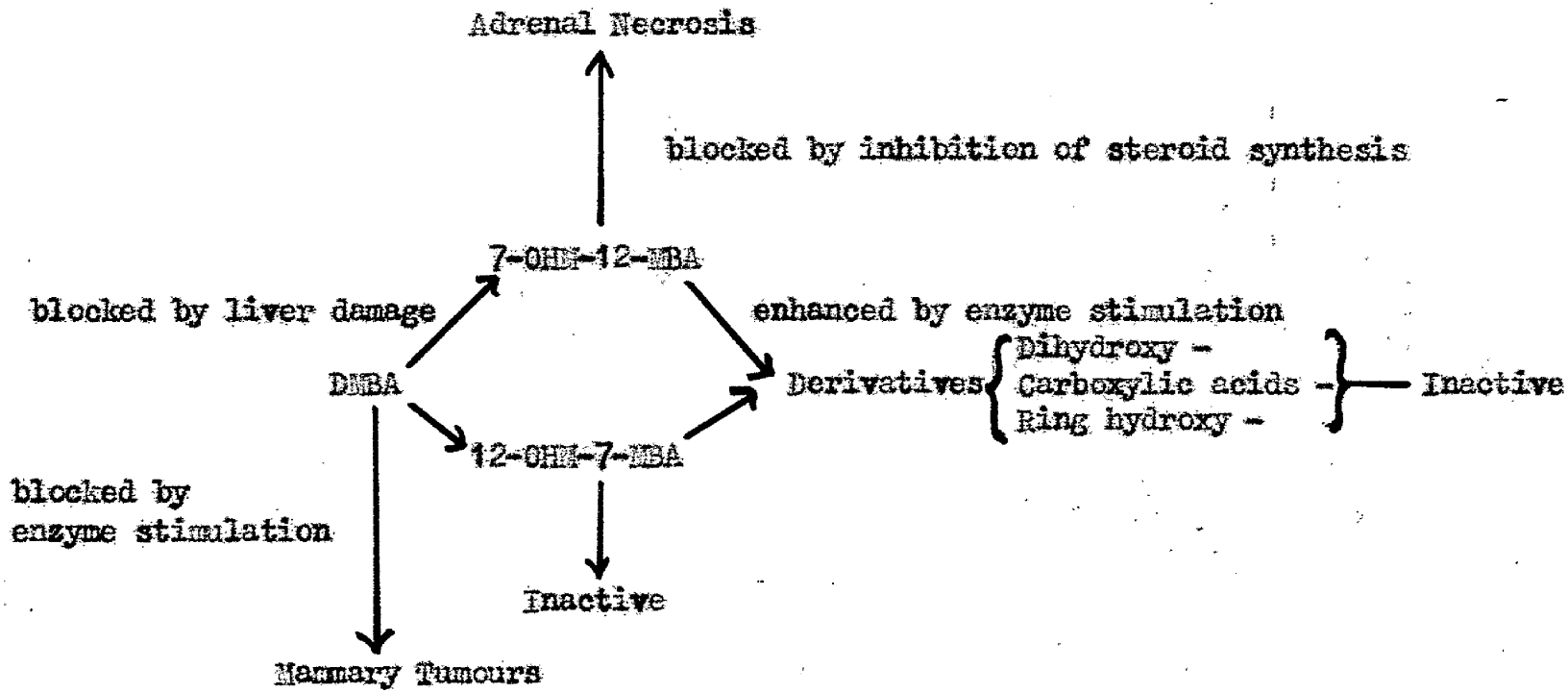
Consideration of the reported biological effects of 7-OHM-12-MBA suggest that this is the metabolite of DMBA

responsible for adrenal damage is illustrated in Figure 27.

The fact that this adrenolytic effect appears to be specific to the rat continues to pose a problem regarding the mechanism by which 7-OHM-12-MBA is able to produce massive adrenal necrosis in this particular species.

FIGURE 27.

Biological Activities of DMBA.



CONCLUSIONS.

This thesis presents a study of the effect of impaired liver metabolism on the adrenolytic and carcinogenic action of DMBA.

The results show:-

1. DMBA does not produce adrenal necrosis, but undergoes metabolism by the liver to form an adrenolytic derivative.
2. DMBA itself, and not its metabolite, is responsible for the induction of mammary tumours.
3. Active synthesis of corticosterone by the adrenal gland is necessary for the development of adrenal necrosis in response to DMBA.

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The original suggestion that the effect of liver interference on the metabolism should be investigated, was made by Dr. D.N. Wheatley. His interest and constructive criticisms proved invaluable.

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